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EVALUATION OF THE DOUBLE DIFFUSION, ENZYME IMMUNOASSAY AND IMMUNOBLOTTING TECHNIQUES, FOR THE DIAGNOSIS OF HUMAN HYDATID DISEASE IN TROPICAL AREAS

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SUMMARY

Hydatid disease in tropical areas poses a serious diagnostic problem due to the high frequence of cross-reactivity with other endemic helminthic infections.

The enzyme-linked-immunosorbent assay (ELISA) and the double diffusion arc 5 showed respectively a sensitivity of 73% and 57% and a specificity of 84-95% and 100%. However, the specificity of ELISA was greatly increased by using ovine serum and phosphorylcholine in the diluent buffer.

The hydatic antigen obtained from ovine cyst fluid showed three main protein bands of 64, 58 and 30 KDa using SDS PAGE and immunoblotting.

Sera from patients with onchocerciasis, cysticercosis, toxocariasis and *Strongyloides* infection cross-reacted with the 64 and 58 KDa bands by immunoblotting. However, none of the analyzed sera recognized the 30 KDa band, that seems to be specific in this assay. The immunoblotting showed a sensitivity of 80% and a specificity of 100% when used to recognize the 30 KDa band.

KEYWORDS: Hydatid Disease; Cross Reactivity; Double Diffusion; ELISA; Immunoblotting.

INTRODUCTION

Hydatid disease constitutes an important health problem throughout the world [10, 18, 20]. Although not showing a high prevalence in the American tropic, it represents a serious diagnostic problem due to serologic cross-reactivity observed with other helminthic infections that share antigens with *Echinococcus*. Diagnosis is usually suspected on epidemiological criteria and clinical characteristics of the cyst lesions. Because in many cases the presence of the parasite is difficult to determine [21, 25], the diagnosis is often confirmed by serological methods. The double diffusion arc 5 test (DD5) and the enzyme linked immunosorbent assay (ELISA) have been widely employed for the diagnosis of human hydatid disease [4, 25]. However, the specificity of these method in tropical areas has not been well established. For this reason, cross reactivity was evaluated herein in patients with onchocerciasis, schistosomiasis, toxocariasis, strongyloidiasis and other helminthic infections. The Western blot technique was also used to analyze the antigenic components responsible for the observed cross reactivity, which permitted as well to evaluate the specificity and sensitivity of this test.

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MATERIALS AND METHODS

Human Serum Specimens

The study included 107 serum samples distributed as follows: (1) 26 patients with confirmed unilocular hydatid disease, 15 from Montevideo/Uruguay and the rest from Venezuela; (2) 61 patients with other helminthic infections: (2a) onchocerciasis (n=16), (2b) cysticercosis (n=10), (2c) schistosomiasis (n=10), (2d) toxocariasis (n=9), (2e) strongyloidiasis (n=7), (2f) hymenolepiasis (n=3), (2g) ascariasis (n=6) and (3) 20 healthy people used as negative controls.

Antigen

A lyophilized antigen was prepared from *E. granulosus* larvae of ovine origin. The antigen was obtained and processed at Instituto de Higiene, Montevideo/Uruguay as described elsewhere [4].

Enzyme linked immunosorbent assay (ELISA-HID)

The ELISA technique was performed following the steps described by COLTORTI [5]. Briefly, Dynatech Immulon/TM2 plates were sensitized overnight at 4°C in a humid chamber with the antigen diluted in carbonate/bicarbonate buffer pH 9.6 to a final concentration of 10μg/ml. The plates were washed with phosphate buffered saline pH 7.2 containing 0.05% Tween 20 (PBS/T 0.05%) and blocked for 15 minutes with PBS/T 0.5%. The microtiter plates were incubated with sera, conjugate and substrate for 1 hour at 37°C in a humid chamber, between steps plates being rinsed three times with PBS/T 0.5%. Alkaline phosphatase labeled goat anti human IgG (Sigma) was used as conjugate and p-nitrophenyl phophate as substrate, diluted in 10% diethanolamine buffer pH 9.8 to a concentration of 1mg/ml. The wells were read on a Labsystem Uniskan II spectrophotometer at 405nm.

The dose-response curve presented as reported by many others [1, 5, 6, 7, 12] an interval of linear response for serum dilution values ranging from 1/64 to 1/1024. So after optimizing the assay, all sera (whole group/ ELISA-HID/G1/n = 107) were studied by diluting 1/256 in PBS/T 0.5% containing 1% BSA, and adding to antigen coated microtiter wells in duplicate. Under these conditions the threshold (D. O. = 0.18) was established as the mean of the negative control sera absorbance values plus 2 standard deviations.

To examine the effect on the specificity of adding ovine serum and phosphorylcholine to the diluting buffer a group of serum samples was selected, consisting of 33 patients with non hydatid infections which showed false reactivity in the ELISA, 23 hydatid patients and 5 healthy controls (ELISA-HID/G2/ n=61). These samples were assayed in duplicate using tris buffered saline (0.15 M NaCL, 0.01 M tris) pH 7.2 containing 0.5% Tween 20.1% ovine serum and 10mM phosphorylcholine (POBS), according to COLTORTI et al [8].

Arc 5 double diffusion test (DD5)

The immunodiffusion assay was performed using the antigen described above and following the method described [4]. Sera from patients with onchocerciasis, cysticercosis, toxocariasis and strongyloidiasis (n=10), previosly positive in the ELISA-HID test were analyzed, as well as negative controls (n=5) and sera from hydatid patients (n=26).

TABLE 1

ELISA and DD5 test sensitivity

Localization of hydatid cyst	Number of individuals	ELISA positive	Test mean O. D.	DD5 positive
Hepatic	15	9	0.461	6
Multiple	3	3	0.386	2
Disseminated	4	4	0.674	4
Pulmonary	1	1	0.363	1
Osseous	2	2	0.549	2
CNS	1	0	0.045	0
Total	26	19	0.413	15
Sensitivity:		(73.33%)		(57.69%)
LC 95:		(56.4% - 90.23%)		(39% - 76.6%)

CNS = Central Nervous System.

Polyacrilamide gel electrophoresis (SDS-Page)

The antigen was run in 10% polyacrilamide gel electrophoresis in reducing conditions using a sample buffer containing 2-mercaptoethanol [14]. Electrophoresis was run at room temperature and constant current of 0.02 A.

Immunoblotting

The electrophoresed antigen was electrontransfered onto 0.45 μm pore size nitrocellulose membranes (Lab. BIORAD) at 4°C for 1 hour, 0.36 A,[24]. After transfer was completed the nitrocellulose membranes were cut into strips which were submerged for 1 hour in a blocking solution: PBS/T 1% containing 5% non fat dried milk (Karla). After incubation, the strips were washed 3 times, 10 minutes each in PBS/T 1% and then reacted with 1ml of the corresponding sera. Following three 10 minutes washes in PBS/T 0.1%, the membranes were incubated with the secondary antibody, peroxidase labeled goat anti human IgG. Finally, the strips were incubated with the substrate for 3 to 4 minutes. The substrate solution was prepared by adding 10mg diaminobenzidine and 10 μl 30% hydrogen peroxidase to 19, 8 ml PBS.

With the purpose of analyzing the antigenic fraction responsible for the cross-reactivity, those samples from patients with non hydatid infections which showed false reactivity in the ELISA-HID were selected (n=11). Samples from hydatid patients (n=15) and healthy controls (n=7) were also studied.

RESULTS

The DD5 and ELISA assays showed a sensitivity of 57.7% and 73.3% respectively (Table 1). The highest sensitivity obtained with both tests corresponded to disseminated, multiple and osseous hydatid diseases (9 positive reactions out of 9), being significantly lower in the case of hepatic hydatidosis (6 to 8 positive reactions out of 15) ($X^2 = 3.91$; $p < 0.05$). The only case of neurological localization was serologically negative.

The specificity achieved with the DD5 assay was 100%, due to the lack of precipitation bands with all the non hydatid sera tested, although some of them cross reacted in the ELISA-HID.

The specificity of the ELISA-HID in healthy individuals was 95% (one positive out 20; LC 95: 90.12%-99.87%). However, in whole group (G1)it was 84% (LC 95: 76.1%-91.9%) and the sensitivity was 73.3% (LC95: 56.4%-90.23%). Cross-reactivity was observed specially with sera from patients with cysticercosis, strongyloidiasis, onchocerciasis, toxocariasis and in the case of onchocerciasis-cysticercosis association (Table 2).

TABLE 2

Specificity of ELISA in the diagnosis of hydatid disease

Non hydatid individuals	Number	Mean D. O.	False positive
Cysticercosis	10	0.101	2
Onchocerciasis	13	0.107	2
Cysti + oncho	3	0.208	3
Strongyloidiasis	7	0.180	3
Toxocariasis	9	0.110	2
Ascariasis	6	0.105	0
Schistosomiasis	10	0.080	0
Hymenolepiasis	3	0.106	0
Healthy	20	0.060	1
Total	81	0.120	13
Specificity:		(84%)	
LC 95:		(76.1% - 91.9%)	

Positive with optical density (O. D.) > 0.18.

In the group which showed false reactivity in the initial ELISA-HID (G2) the sensitivity was 74% and the specificity 60.5% (Table 3). Adding ovine serum and phosphorylcholine to the diluting buffer did not affect the sensitivity of the test, however the number of false positives decreased nearly 50% (from 15 to 8 false positive reactions), increasing the specificity of the assay to 79%. The reduction of cross reactivity was

TABLE 3

Results of ELISA for hydatid disease using different diluents

Individuals	Number	Positives PBS	Positives POBS
Hydatid Patients Sensitivity	23	17 (74%)	17 (74%)
Non hydatid individuals			
Onchocerciasis	10	6	3
Cysticercosis	7	2	2
Toxocariasis	9	3	0
Strongyloidiasis	7	4	3
Healthy	5	0	0
Total	38	15	8
Specificity:		(60.52%)	(78.95%)

PBS: phosphate saline solution.
POBS: (phosphorylcholine + ovine serum) phosphate saline solution.

significative in the cases of toxocariasis and onchocerciasis, but less evident in the cases of strongyloidiasis and cysticercosis.

When the ovine antigen was examined by electrophoresis, three protein bands were demonstrated, corresponding to molecular weights of 68, 54 KDa and a less intense 30 KDa band. All sera from hydatid patients recognized at least one of the identified bands, while the healthy controls did not react with any of them (Table 4). When sera from patients with non hydatid helminthic infections cross-reacting in the ELISA-HID were examined, it became clear that 61.1% of them recognized the 58 KDa band and the remaining 33.3% the 64 KDa polypeptide. However, none of these non hydatid sera reacted with the 30 KDa band, yielding 100% specificity. However, this band was recognized by most (80%) of the hydatid sera.

DISCUSSION

Autoctonous polycystic hydatid disease caused by either *Echinococcus vogeli* or *E. oligarthrus* have been described in American tropical areas [13,20,21]. However all the patients from Venezuela studied by us, proved to be cases of unilocular hydatid disease, so this paper will deal only with *E. granulosus* infection.

The DD5 test showed a high specificity but a poor sensitivity, as discussed previously by COLTORTI et al [5,7]. The use of DD5 as a screening test in tropical areas is limited by the fact that it is a time consuming technique and requires high antigen quantities.

Although the ELISA-HID detected a significantly higher number of hydatid cases, 26% of confirmed cases resulted negative. This has been attributed to anatomical barriers (the barrier of the hydatid cyst, or the hematoencephalic barrier in the case of neurological hydatidosis [20]) or the formation of immunocomplexes which decrease the percentage of individuals with free antibodies [9]. The role of these barriers was confirmed by the higher sensitivity of the test in situations where the adventitious capsule is not formed (osseous hydatid disease) or when the cyst membrane is broken (disseminated disease) [20].

The sensitivity and specificity of the DD5 test and ELISA-HID determined in this study were low in comparison to those reported by others in non tropical endemic areas of the continent [2,18]. The lower specificity found may be accounted for by the control population studied, which included different proportions of patients with other helminthic infections. The diseases which showed most cross-reactivity were cysticercosis, strongyloidiasis, toxocariasis and onchocerciasis, which have in common the long persistence or repeated migrations of larvae in the host tissues. On the other hand, the fact that schistosomiasis did not react positively, would be in agreement with a previous work [22]. In contrast, the immunoblotting technique showed a high specificity (100%) and sensitivity (80%), reported as well by SIRACUSANO et al [22]. Some of the diseases responsible for the observed cross-reactivity are of focal transmission in rural areas (onchocerciasis, strongyloidiasis) while others have a more spread distribution, including urban areas. In this regard, epidemiology could help to establish the significance of a positive serology in a particular patient and also contribute to the causal diagnosis.

TABLE 4

Recognition of hydatic antigenic fractions by immunoblotting

Individuals	Number of Samples	KDa Bands recognized		
		30	58	64
Hydatid Patients	15	12	15	14
Non Hydatid Individuals				
Onchocerciasis	3	0	3	2
Strongyloidiasis	2	0	2	2
Cysticercosis	3	0	3	0
Toxocariasis	3	0	3	2
Healthy	7	0	0	0
Total	18	0	11	6
Specificity:		100%	61.11%	33.33%

The cross-reactivity observed in this study could depend on the presence of antigens shared by *Echinococcus granulosus* and other agents of helminth infections or erythrocytes.

The hydatid antigen of ovine origin showed three bands of 64, 58 and 30 KD in reducing conditions. However, it is difficult to establish the correspondence of the polypeptides identified in this study with the major antigenic components of the hydatid liquid, antigen B[19] and antigen 5 reported by CAPRON [21, 26]. In fact, although in non reducing conditions antigen 5 presents a molecular weight of 67 KD, after reduction two subunits of 24 and 38 KD are found [15]. In these conditions, SIRACUSANO et al.[22] reported a high specificity for the 39 KDa band which nevertheless cross-reacted with various helminthic infections in the study of SHEPHERD & McMANUS [23], due to the presence of a phosphorylcholine epitope. Such epitope is also found in red blood cell surface carbohydrates, against which a spontaneous, normal immune response can be observed [3]. This antigenic determinant was probably present in the 58-64 KDa bands identified in this study, because their cross reactivity could be considerably reduced when incubating sera with POBS. This effect reported previously[8] is of particular interest in relation to toxocariasis which could share common risk factors with hydatidosis.

Nevertheless the cross-reactivity was not reduced in the cases of onchocerciasis, cysticercosis and strongyloidiasis. This indicates the presence of other shared epitopes. In this sense it is interesting the high specificity observed with the 30 KDa band. Some authors [17, 23], attributed the highest specificity to the components of antigen B corresponding to relative molecular weights of 8, 16, 24 and 32 KDa [15], but these findings are controversial [22]. Studies in progress are oriented to the characterization of the 30 KDa band and analysis of its specificity by increasing the number of samples from patients with helminthic infections.

RESUMEN

Evaluación de las técnicas de Doble Difusión, Ensayo Inmunoenzimático e Inmunoblotting en el diagnóstico de la hidatidosis humana en áreas tropicales.

La Hidatidosis en áreas tropicales representa un serio problema diagnóstico por la alta frecuencia de reactividad cruzada con otras infecciones helmínticas. Las técnicas de inmunoensayo enzimático (ELISA) y doble difusión arco 5 (DD5) mostraron una sensibilidad de 73 y 57 % y una especificidad de 84-95% y 100%, respectivamente. La especificidad en la técnica de ELISA, fue mejorada sustancialmente al emplear como diluyente de los sueros una solución buffer conteniendo suero ovino normal y fosforilcolina.

En líquido obtenido de hidátides de *Echinococcus granulosus* de origen ovino, se demostraron tres bandas de origen proteico de 64, 58 y 30 KDa de peso molecular, empleando SDS e inmunoblotting.

Sueros de pacientes con estrongiloidiasis, oncocercosis, toxocariasis y cisticercosis reaccionaron con las bandas de 64 y 58 KDa en inmunoblotting. Sin embargo ninguno de los sueros reconoció la banda de 30 KD, la cual parece ser específica para este ensayo. La técnica de inmunobloting empleada para poner en evidencia la banda de 30 KDa con fines diagnósticos, exhibió una sensibilidad de 80% y una especificidad de 100%.

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