SPECIFIC DIAGNOSTIC ANTIGENS OF *ECHINOCOCCUS GRANULOSUS* DETECTED BY WESTERN BLOT

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Summary :

A western blot assay was performed for the detection of *Echinococcus granulosus* specific antigens useful for the diagnostic of hydatic disease. 191 sera were tested, 105 coming from patients with different localizations of hydatic cysts and 86 from persons either healthy or presenting other diseases. 48 different antigenic bands were detected using sera from patients with hydatidosis. A 35 kDa antigen co-migrating with a band labeled by a McAb specific of antigen 5 was recognized in western blot by only 68% of the sera able to precipitate antigen 5 in immunoelectrophoresis. A 8 kDa antigen corresponding to the specific *E. granulosus* antigen already described has been recognized by 80% of the sera coming from patients with hydatidosis and not by the 86 control sera. Bands of 21, 30, and 92 kDa appeared also specific and were recognized by at least 50% of tested sera. These antigens appeared unrelated one to each other. 103 out of the 105 sera from patients with hydatidosis were able to recognize at least one of the 8, 21, 30, 35 or 92 kDa specific antigens. The present results suggest that western blot could be useful for the diagnosis of hydatidosis as far as the criteria of positivity is based on the recognition of at least one of the major specific antigens.

KEY WORDS : hydatidosis. western blot. immunoelectrophoresis. indirect hemagglutination. *Echinococcus granulosus*.

Résumé : DÉTECTION DES ANTIGÈNES SPÉCIFIQUES D'*ECHINOCOCCUS GRANULOSUS* PAR LA TECHNIQUE D'IMMUNOBLOT

La technique d'immunoblot fut adaptée au diagnostic de l'hydatidose par la détection d'antigènes spécifiques. 191 sérums furent testés, 105 provenant de patients présentant différentes localisations de kyste hydatique et 86 sérums de sujets sains ou atteints d'autres maladies. 48 bandes de masses moléculaires différentes furent détectées par les sérums de sujets atteints de la maladie hydatique. La révélation par l'anticorps monoclonal (McAb) a permis de mettre en évidence une bande antigénique de 35 kDa. Cette bande a été observée dans 68% des sérums de sujets ayant présenté l'arc 5 en immunoélectrophorèse. Une seconde bande de 8 kDa, déjà décrite, a été reconnue par 80% des sérums de patients hydatiques. Aucun sérum de sujets témoins ne reconnaissait cette bande. D'autres bandes (21, 30, 92 kDa) apparaissaient également spécifiques, et reconnues par au moins 50% des sérums testés. 103 sérums parmi les 105 étudiés reconnaissaient au moins une des cinq bandes majeures. En admettant comme critère de positivité l'apparition d'au moins une de ces bandes majeures, la technique d'immunoblot serait très utile pour le diagnostic de cette parasitose.

MOTS CLÉS : *hydatidose. western blot. immunoelectrophorèse. hémagglutination indirecte.* Echinococcus granulosus.

INTRODUCTION

The diagnosis of hydatidosis is strongly helped by the detection of antibodies in the sera of patients using either quantitative (e.g. immunofluorescence or hemagglutination assays) or qualitative methods (e.g. immunoelectrophoresis, counter-immunoelectrophoresis). The quantitative methods are known for their high sensitivity but their relatively poor specificity due to various cross reactions with antigenic components of other cestods or non cestod helminths (Rickard *et al.*, 1977; Ambroise-Thomas *et al.*, 1984; Pezella *et al.*, 1984; Shepherd *et al.*, 1987; Al Yaman *et al.*, 1989). On the contrary, the specificity of qualitative methods is better since these tests are based on the recognition of specific antigens. In this respect, the recognition of arc 5 by immunoelectrophoresis has been extensively used for the diagnosis of hydatidosis in spite of its relatively low sensitivity (Capron *et al.*, 1968; Bout *et al.*, 1974). A second antigen of diagnosis importance called antigen B (Oriol *et al.*, 1971) or antigen 4 (Puzzuolli *et al.*, 1975) has also been described, but is not often used.

More recently, taking advantage of sensitivity gain offered by the western blot (WB) assay, this qualitative technique has been adapted for the diagnosis of hydatid disease (Xue *et al.*, 1987). By this method it has been possible to detect antibodies against antigen 5 (Di Felice *et al.*, 1986; Baba, 1987; Gottstein *et al.*, 1987) and also against a 8 kDa antigen (Maddison *et al.*, 1989).

The present study has been designed to investigate

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Healthy subjects[a]	25, 34, 40, 64, 96
Anisakiasis	34, 40
Candidiasis	25, 34, 40, 48, 50, 62, 98
Cysticercosis	26, 36, 40, 41, 43, 47, 49, 57, 60, 79, 98, 107
Fascioliasis	24, 25, 34, 36, 38, 48, 50, 64, 75, 100, 104, 115
Filariasis	24, 25, 34, 36, 48, 50, 64, 72, 94, 96, 100, 104, 115
Hepatitis	32, 34, 37, 40, 50, 64, 104
Malaria	26, 32, 34, 37, 40, 50, 64, 104
Schistosomiasis	37, 48, 50, 64
Strongyloidiasis	24, 25, 34, 36, 38, 40, 48, 50, 64, 75, 94, 96, 100, 104, 115
Taeniasis	34, 48, 50, 64, 75
Toxoplasmosis	32, 34, 37, 40, 50, 64, 104
Trypanosomiasis	34, 37, 64, 104
Visceral larva migrans	34, 40, 65
Visceral leishmaniasis	34, 50, 64, 104

a : Weak reactions, never observed together and in 4/9 of tested sera.

Table I. – Distribution of the antigenic bands (expressed in kDa) revealed by sera of patients without hydatidosis.

band m.w. (kDa)	92	35	30	21	8
hydatidosis (+) n=105	50	68	73	69	80
hydatidosis (-) n=86	0	0	0	0	0

m.w. : molecular weight

Table II. – Percentage of sera able to recognize the 92, 35, 30, 21 or 8 kDa bands.

number of specific bands labeled	0	1	2	3	4	5
number of responding sera	2	7	10	34	26	26
cumulative number of positive sera		7	17	51	77	103
% of responding sera	1.9	6.7	9.5	32.3	24.8	24.8
cumulative % of positive sera		6.7	16.2	48.5	73.3	98.1

Table III. – Sera from patients with hydatidosis (n=105) able to recognize from 0 to all of the 5 specific antigens (92, 35, 30, 21 or 8 kDa bands).

whether other antigens than fraction 5 and the 8 kDa antigen could be specifically recognized by sera from patients infected by *E. granulosus*. Moreover, we deliberately used a commercial antigen in order to let the possibility to different laboratories to easily copy our procedure. We report the recognition of specific antigenic fractions of diagnostic interest and a method of lecture that could increase the sensitivity of the assay.

MATERIALS AND METHODS

HUMAN SERA

105 serum specimens coming from patients with surgically confirmed *E. granulosus* infections were investigated. All patients have been regularly living in Tunisia. The anatomic distribution of the 105 cysts was as follows : 78 hepatic, 22 pulmonary, three bone and two pericardial cysts. Sera were stored at - 20 °C before testing.

86 sera were included in the study as controls : nine from healthy subjects, 10 with candidiasis, four with visceral *Larva migrans* (toxocariasis or anisakiasis), six with filariasis (*Loa loa*), two with strongyloidiasis, 14 with cysticercosis, one with taeniasis (*Taenia saginata*), six with fascioliasis (*Fasciola hepatica*), two with schistosomiasis (*Schistosoma mansoni*), nine with toxoplasmosis, five with trypanosomiasis (four with *Trypanosoma cruzi* and one with *Trypanosoma gambiense*), two with visceral leishmaniasis, seven with malaria (*Plasmodium falciparum*) and nine with viral hepatitis.

SPECIFIC REAGENTS

Antigen purchased from Bio-Mérieux (Marcy l'Étoile, France) was used for both WB (5mg/ml in Tris HCl buffer, 0.5M, pH 6.8, 20% SDS), and immunoelectrophoresis. This company prepared the antigen from sheep hydatid cyst fluid.

The monoclonal antibody (McAb) Bio 225-840 specific of *E. granulosus* antigen 5 was used as a control (Biosoft, Paris, France).

SDS-PAGE AND WESTERN BLOT

Electrophoretic separation of the hydatic antigens was performed under reducing conditions with a 15% acrylamide separating gel (Laemmli, 1970). Antigens were treated 5 min at 95°C in reducing conditions and submitted to electrophoresis (250µg/cm). Antigenic components were blotted onto nitrocellulose paper (Gottstein *et al.*, 1987) and probed with 2 µl of each serum specimen diluted in 4 ml of Tris NaCl buffer pH8 with 1% skimmed milk. The antigen-antibody complexes were revealed by anti-human IgG antibodies labeled with alkaline phosphatase (Promega, S 3821) and treated with BCIP-NBT (Promega, S 3771) (Verastegui *et al.*, 1992).

Positive control performed with McAb was revealed using goat anti-mouse IgG (H+L) labeled with alkaline phosphatase (S 3721). Molecular weight (m.w) of the antigens were determined from m.w. standards (14-94 kDa, Pharmacia) and also by comparison with the bands recognized by a positive human sera with high antibody levels together with the McAb Bio 225-840. This monoclonal labeled a major 35 kDa band and weakly some other bands of minor m.w.

INDIRECT HEMAGGLUTINATION ASSAY (IHA) AND IMMUNOELECTROPHORESIS (IEP)

Fumouze kit (Fumouze, Clichy, France) was used for the detection of anti-*E. granulosus* antibodies by IHA. IEP was performed as previously described (Bout *et al.*, 1974; Ambroise-Thomas *et al.*, 1984). All sera from patients with hydatidosis were positive with at least one precipiting band in IEP and with an IHA titer equal or more than 160.

RESULTS

A total of 48 different antigenic bands with m.w. ranging from 8 to 120 kDa were revealed with the 105 sera coming from *E. granulosus* infected patients. Less than 17 different bands were labeled with the 86 sera coming from individuals without hydatidosis. Sera from patients with taeniasis labeled up to five different bands and with cysticercosis up to 12. Only some sera from healthy subjects recognized five different bands. Surprisingly patients with fascioliasis labeled 12 different bands and with strongyloidiasis 15 (table I).

27 antigenic bands of different m.w. (ranging from 120 to 8 kDa) were recognized by sera from patients with hydatidosis but not by sera from patients without hydatidosis. Among them, only bands of 92, 35, 30, 21 or 8 kDa were recognized by at least 50% of the sera tested (table II). The labelling of the 22 other bands occurred with a frequency of less than 44 %.

The 35 kDa specific antigen, co-migrating with the antigen labeled by the McAb was recognized in WB by only 68% of the 105 sera from patients with hydatidosis while all these sera were able to precipitate the antigen 5 in IEP performed with the same antigen (fig. 1).

A specific 8 kDa antigen which could correspond to the one described by Maddison *et al.*, 1989, has been identified during our experiments. 80 out of the 105

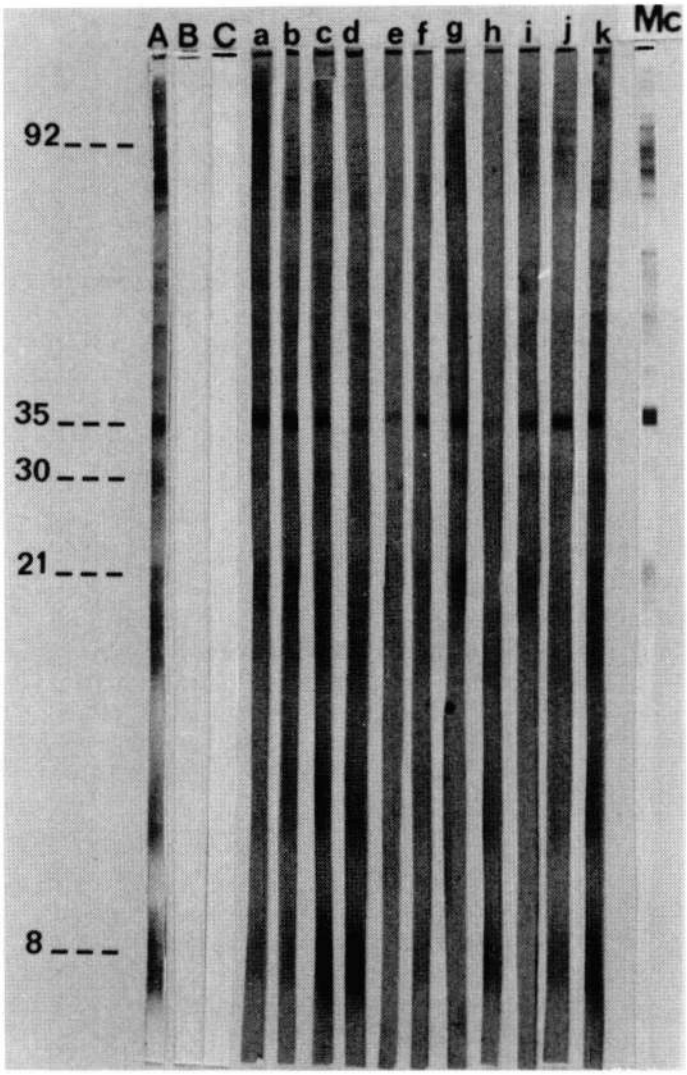

Fig. 1. – Antigenic bands observed in serum samples from patients with hydatidosis.
Lane A : Positive control serum . Lane B : Healthy subject serum. Lane C : Conjugate control. Lanes a-k : differents sera from patients with hydatic disease. Mc : serum McAb (monoclonal reference).

sera coming from patients with hydatidosis were able to label this band.

Results were also expressed considering as specifically positive sera able to label at least one of the 92, 35, 30, 21 or 8 kDa bands. In this case, 103 out of the 105 (98.1%) sera were considered as positive (table III).

DISCUSSION

Using sera from patients with or without hydatidosis we were able to identify by WB antigenic components from hydatid cyst fluid which could be useful for the diagnosis of human hydatidosis. 27 antigenic bands were specifically recognized by sera from *E. granulosus* infected patients but with a low frequency for most of them. On the contrary, antibodies against the 92, 35, 30, 21 and 8 kDa specific antigens were detected in at least 50% of the tested sera.

It is possible that among the 27 antigenic bands detected some can correspond to processed fragments coming from one particular molecule. However, antibodies against the 92, 35, 30, 21 and 8 kDa specific antigens did not appear associated which suggests that the corresponding antigenic fractions are independent one from each other. Moreover, the 35 and 8 kDa antigens are probably unrelated since our observations suggest they are respectively corresponding to 2 different antigens : antigen 5 (Capron *et al.*, 1968; Capron *et al.*, 1970; Bout *et al.*, 1974) and the 8 kDa antigen described by Maddison *et al.*, 1989.

Percentage of detection of antibodies against the 92, 35, 30, 21 and 8 kDa specific antigens, ranging from 50 to 80 %, does not probably reflect sensitivity (number of positive/number sera from infected patients) of the assay. Sera used in the present study were corresponding to patients with major clinical evidences of evolutive disease. These sensitivity percentages should be lower in a population of patients routinely analysed. Moreover, our deliberate selection of patients together with the fact that were not considered sera with an absence of antibodies against either antigen 5 in IEP or in IHA, does not allow a comparative study of the specificity or sensitivity between the three different serologic assays.

Antibodies against the 8 kDa antigen were detected in 80% of sera tested which means that using this criteria for diagnostic purpose, 21 out of the 105 patients with hydatidosis would be considered as negative. This observation is in concordance with results previously reported by Maddison *et al.* However, the percentage of false negative decrease to two out of 105 with a specific diagnosis based on the recognition of at least one of the 92, 35, 30, 21 and 8 kDa specific antigens.

We observed a marked dissociation between results of detection of antibodies against antigen 5 in IEP and WB. This could be explained by a partial conformational modification of antigen 5 (migrating as a 35 kDa antigen in WB) during SDS-PAGE and transfer onto nitrocellulose. In this case, antibodies against SDS or boiling-sensitive epitopes could not be detected. Additional experiments will be necessary to compare the sensitivity of IEP and WB assays using sera negative in IEP coming from *E. granulosus* infected patients, which was not the case in the present study.

The detection of antibodies against numerous *E. granulosus* antigen by WB in patients without hydatidosis as well as the recognition of various *E. granulosus* antigens out of the 92, 35, 30, 21 and 8 kDa specific antigens by sera from patients with hydatidosis make tricky the use of this assay. However, with a little bit of practice and the use of reference controls (standard human sera, McAb against the 35 kDa antigen) the WB assay can be used in routine all the more so since the antigen and the McAb are commercially available.

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