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Immunochemical analysis of *Echinococcus granulosus* and *Echinococcus multilocularis* antigens

Ľ. TURČEKOVÁ, J. KINČEKOVÁ, M. REGENSBOGENOVÁ[1], K. REITEROVÁ, P. DUBINSKÝ

Parasitological Institute of the Slovak Academy of Sciences, Hlinkova 3, 040 01 Košice, Slovak Republic, E-mail: *turcela@saske.sk*; [1]P. J. Šafárik University, Faculty of Natural Sciences, Department of Molecular and Cellular Biology, Moyzesova 11, 041 67 Košice, Slovak Republic

Summary

The protein profile of three antigens of *Echinococcus* spp. was characterised by electrophoretic methods in SDS-PAGE. The composition of somatic antigens of protoscolices and hydatid fluid of *Echinococcus granulosus* and somatic antigens of *Echinococus multilocularis* protoscolices was qualitatively compared. Immunoblot analysis of patient sera and ELISA method confirmed cystic echinococcosis and showed that somatic antigen of *E. granulosus* is less suitable for diagnosis of echinococcosis, due to insufficient number of unique features manifested. Hydatid fluid of *E. granulosus* showed a high sensitivity for diagnosis of cystic echinococcosis. Immunoblot analysis of fluid showed the presence of antigen subunit 5 in all patient sera positive for cystic echinococcosis. Unlike this, the composition of antigen B consisting of 24 kDa, 16 kDa and 8 kDa protein fractions, varied considerably. The protein with a 8 kDa molecular weight, representing a genus-specific characteristics for *Echinococcus* spp., was consistently detected in hydatid fluid of all positive patients.

Key words: *Echinococcus granulosus*; *E. multilocularis*; antigens; human sera; immunodiagnosis

Introduction

Echinococcosis is helminthic zoonosis affecting humans and animals. It is mainly caused by *E. granulosus* (Batch, 1986) and *E. multilocularis* cestodes (Leuckart, 1863). Carnivores, particularly dogs and foxes, serve as definitive hosts of these parasites. Intermediate hosts, in which the metacestode stage develops, are represented by a variety of farm animals and feral animals, several micromammal species, as well as humans. Intermediate hosts are infected perorally with eggs, which are disseminated via faeces of definitive hosts into the environment. The geographical position of Slovakia provides suitable conditions for transmission and spread of this zoonosis which presents a topical health and veterinary problem.

Clinical signs of cystic and alveolar echinococcoses may imitate liver diseases such as hepatic carcinoma and their clinical diagnosis is thus often problematic. Apart from depictive methods ultrasonic examination (USG), computer tomography (CT) the use of serological methods is necessary to confirm the presence of parasitic cestodes. At present antigen B detectable by immunoelectrophoretic methods is being used for diagnosis of cystic echinococcosis as it appears in human sera after infection.

The aim of the study was to compare electrophoretically three different antigens of *Echinococcus* spp. and to characterise their diagnostic efficiency with the use of patient sera.

Materials and Methods

The somatic antigen of *E. granulosus* was prepared from the fertile liver cysts recovered from pigs in Slovakia. The isolated protoscolices were washed twice and ultrasonicated in PBS buffer (pH 7.2) under cooling conditions at 4 – 6°C during 30 min (60 % of efficiency) with Ultrasonic Dismembrator 300 W (Dynatech). The homogenate was centrifuged at 15 000 *g* and supernatant was taken as antigen. Hydatid fluid was isolated from same liver cysts. *E. multilocularis* metacestodes were maintained in Mongolian gerbils (*Meriones unguiculatus*) by intraperitoneal passage of protoscolices. For the preparation of antigen, *E. multilocularis* metacestodes were taken from the peritoneal cavity of gerbils infected four months before (Auer *et al.*, 1988). Somatic antigen of *E. multilocularis* was prepared by homogenization of metacestodes and isolated protoscolices were ultrasonicated in PBS as described above. The antigens were dialyzed against PBS (pH 7.2) (Dialysis tube for molecular weight 6 000 or greater, Sigma) and lyophilized.

Hyperimmune rabbit sera were prepared from the New Zealand white rabbits weighting 3 kg. The sera were inoculated with antigens of *E. granulosus* and *E. multilocularis* using Freund's adjuvant in 3 doses at one-week intervals (Turčeková *et al.*, 1997).

Sera for analyses were obtained from 13 patients with suspected echinococcosis as well as from patients with cysts of unknown origin. Serological examinations were also performed in patients with some liver or lung disorders without any cystic findings. Prior to examination, all sera were frozen at -20°C without preservation. The sera were investigated by enzyme-linked immunosorbent assay (ELISA).

SDS-PAGE electrophoresis was performed using a Bio-Rad Mini Protean Slab Cell on a 12 % polyacrylamide gel and 4 % stacking gel was cast basically following the method of Laemmli (1970). The antigens were electrophoresed at 200 V for approximately 50 min at room temperature. For qualitative evaluations of proteins the gels were stained with 0.1 % Coomassie brilliant blue according to Trah and Schleyer (1982).

After SDS-PAGE electrophoresis, the parallel gels were used for immunological study. Proteins were blotted onto nitrocellulose membrane (NC) in Tris-glycine buffer (pH 8.8) at constant voltage of 250 V for 1 h. After blotting, the NC membrane was blocked with 5 % nonfat milk in PBS (pH 7.2) for 2 h. The nitrocellulose membrane was cut into 3 mm wide strips and incubated with rabbit hyperimmune sera diluted 1:50 in 3 % nonfat milk in PBS for 1 h. The strips were washed three times with PBS-Tween 20 and reacted with hyperimmune sera with SwAR/IgG (swine-anti rabbit IgG peroxidase conjugate) or with patient sera SwAHU/IgG (swine-anti humane IgG peroxidase conjugate) (1:300) for 2 h at room temperature with shaking. The strips were washed again three times with PBS-Tween 20 and bands were developed in 0.05 % diaminobenzidine-tetrahydrochloride and 0.03 % hydrogen peroxide (Mouneimne *et al.*, 1997).

Results

Electrophoretic analysis (SDS-PAGE) of proteins involved in sonicated antigen from protoscolices and hydatid fluids of *E. granulosus* and sonicated antigen from protoscolices of *E. multilocularis* pointed out to the significant qualitative differences in the protein spectrum of studied antigens (Fig. 1).

Protoscolices antigen of *E. granulosus* has been characterised by 5 bands with the molecular weights of 65 kDa, 57 kDa, 52 kDa, 40 kDa a 38 kDa. In the protein profile of hydatid fluid, 14 bands ranging from 200 kDa to 8 kDa were detected. The significant differences in the occurrence of some proteins were detected within the zones of 66 kDa – 52 kDa, and 38 kDa – 24 kDa. The protein profile in the hydatid fluid was more similar to the content of proteins in the protoscolices antigen of *E. multilocularis* than to the protoscolices antigen of *E. granulosus*. The differences between the two former antigens were attributed to the absence of proteins with the molecular weights of 52 kDa, 38 kDa and 35 kDa, and the presence of the 16 kDa protein. These antigens were used for examination of patient sera by Western blot analysis.

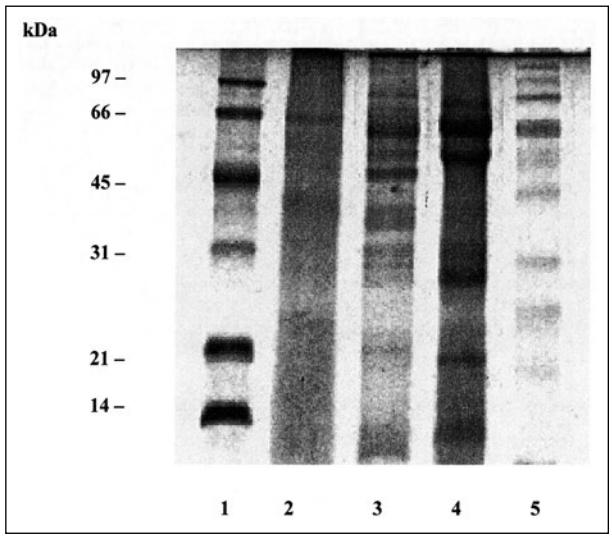

Fig. 1. Electrophoretic diagram of proteins from three different antigens of *Echinococcus* spp. examined in 12 % SDS-PAGE gel. The proteins were stained with Coomassie brilliant blue. 1 – marker (BioRad); 2 – sonicated antigen prepared from *E. granulosus* protoscolices; 3 – hydatid fluid of *E. granulosus*; 4 – sonicated antigen prepared from *E. multilocularis* protoscolices; 5 – marker (Gibco)

Table 1. Clinical and serological findings of investigated patients

Organs	No. of patients	ELISA titers	USG/CT findings	Surgical investigation	Western blot analysis
Liver	3	1:400	positive	+	+
	4	1:800	positive	+	+
	5	1:400	positive	+	+
	6	1:1200	positive	+	+
	7	1:800	positive	+	+
	8	1:1600	positive	+	+
	9	1:400	positive	+	+
	10	1:1200	negative	not done	+
	11	1:400	negative	not done	+
	12	1:400	negative	not done	-
Lung	13	1:400	negative	not done	-
	14	1:800	negative	not done	-
	15	1:400	negative	not done	-

For immunodiagnosis, samples from patients suspected for echinococcosis (confirmed by the ELISA pre-screening) were divided into three groups (Table 1). The first group comprised patients with the surgically confirmed *E. granulosus* cysts (samples 3 – 9). The second group consisted of seropositive patients in which, however, USG and CT of the liver did not confirm cystic structures (samples 10 – 12). The third group included patients with the sample

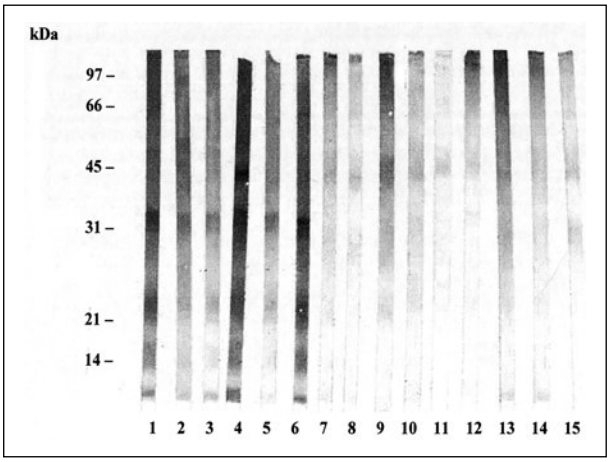

Fig. 2. The immunoblot analysis of sonicated protoscolices antigen of *E. granulosus* with *Echinococcus*-positive human sera. 1 – positive control of *E. granulosus* with hyperimmune rabbit sera; 2 – positive control of rabbit hyperimmune sera of *E. multilocularis*; 3, 4, 5, 6, 7, 8, 9 – positive surgical finding of *Echinococcus* spp.; 10, 11, 12 – without USG and CT finding in liver; 13, 14, 15 – without USG and CT findings in lungs

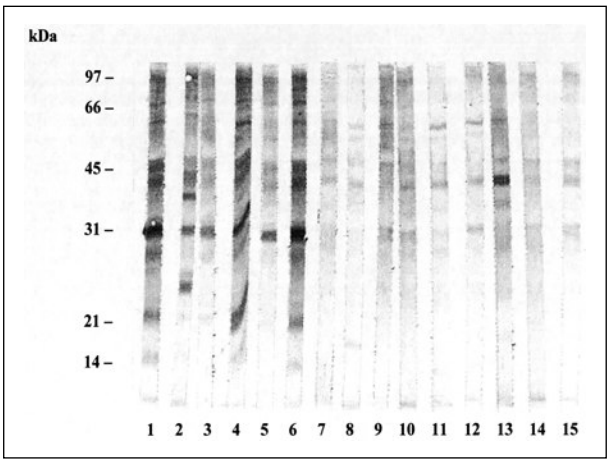

Fig. 3. The immunoblot analysis of hydatid fluid of *E. granulosus* with *Echinococcus*-positive human sera. 1 – positive control of *E. granulosus* with hyperimmune rabbit sera; 2 – positive control of rabbit hyperimmune sera of *E. multilocularis*; 3, 4, 5, 6, 7, 8, 9 – positive surgical finding of *Echinococcus* spp.; 10, 11, 12 – without USG and CT finding in liver; 13, 14, 15 – without USG and CT findings in lungs

numbers of 13 – 15, with suspected findings in lungs without USG and CT confirmation.

Immunoblot profile of sera with protoscolices antigen of *E. granulosus* was not sufficiently expressed. In samples 8, 11, 12, 13, 14 and 15, the genus-specific fragment 8 kDa was not detected either (Fig. 2). In comparison with the positive *E. granulosus* control, samples 11 and 15 showed an absence of bands with molecular weights of 35 kDa and 38 kDa.

Immunoblot analysis of hydatid fluid of *E. granulosus* with patient sera was more sensitive than analysis with protoscolices antigen (Fig. 3). In all patients' sera, antigen subunit 5 of molecular weight 38 kDa was detected along with antigen B, consisting of fractions 24 kDa, 16 kDa and 8 kDa. The genus-specific band of molecular weight 8 kDa was not detected in samples 12, 13, 14 and 15, in which also some antigen-B fractions were absent.

Positive hyperimmune rabbit sera served as control group; using them we were able to detect fragments with 62 kDa and 54 kDa molecular weights, typical for alveolar echinococcosis. These fragments were constantly absent in antigens of *E. granulosus* (Figs. 2 and 3).

Discussion

Clinical manifestation of larval echinococcosis varies and it is implicated by localization of cysts, their size and age. The duration of clinical manifestation after infection also varies and it often takes several years. Most diseases are diagnosed in humans aged from 10 to 50 years (Schantz and Gottstein, 1986). Diagnosis of *E. granulosus* is rather complicated given the wide protein spectrum being available, which is probably mainly influenced by large number of intermediate hosts. Within the intraspecific variability of *E. granulosus*, 10 strains have been so far described characterised by various pathogenicity (McManus and Thompson, 2003; Lavikainen *et al.*, 2003).

Using the electrophoretic separation of proteins and Western blot analysis, it can be obtained a broad spectrum of antigen subunits followed by protein antigen-antibody specific complex. The electrophoretic profile of our ultrasonicated protoscolices antigen of *E. granulosus* has considerably differed from the protein profile of hydatid fluid, which was characterised by a larger amount of proteins. Electrophoretic profiles of both *E. granulosus* antigens and somatic antigen of *E. multilocularis* were composed of the two significant protein groups. These groups have been already described as antigen 5 and antigen B. Antigen 5 comprises two large subunits of molecular weights of 38 kDa and 28 kDa (Lightowlers *et al.*, 1989). Shepherd and McManus (1987) defined the antigen of low molecular weight 8 – 30 kDa, and named it antigen B. The use of SDS-PAGE and following immunoblot analysis has resulted in detecting a specific diagnostic feature of molecular weight 8 kDa, which eliminates cross-reactions (Maddison *et al.*, 1989). The findings of several authors have proved that only antigen B is the genus-specific (8 kDa) (Ito *et al.*, 1999). Antigen B, a subunit of the *E. granulosus* hydatid fluid, contains proteins of 8 kDa, 16 kDa, and 24 kDa molecular weights.

Immunoblot analysis of patient sera with hydatid fluid, which was confirmed by ELISA test as positive for cystic echinococcosis, showed in contrast to the immunoblot analysis of *E. granulosus* somatic antigen a considerable variability in the composition of antigen B. In this aspect, the results are similar to those of Siracusano *et al.* (1991) and Craig (1993). We considered all patients with the detected protein of 8 kDa molecular weight as positive for cystic echinococcosis. Only in the two cases the subunit of antigen B was not detected – in patients sera with the negative

USG and CT findings in liver and lungs. Despite the fact that ELISA test has remained positive in these patients, Western blot analysis and imagery has pointed to other than the echinococcosis finding. Cross-reactions are often caused with other infections and/or degenerative disorders (Gottstein, 1992).

For the genus-specific differentiation of *E. multilocularis*, Auer *et al.* (1988) reported a specific protein of molecular weight 62 kDa and Gottstein (1992) reported a presence of the protein of 54 kDa. The alveolar echinococcosis has not been detected in sera of analysed patients. This disease had been confirmed for the first time in Slovakia in female patient originated from a locality with a high prevalence of this cestode in red foxes (Kinčeková *et al.*, 2001; Miterpáková *et al.*, 2003). The second case of autochtonous alveolar echinococcosis in Slovakia was accordingly detected in this locality (Kinčeková *et al.*, 2002).

In spite of numerous efforts to develop highly sensitive and specific methods, indirect serological diagnostics are not uniform. Ersfeld *et al.* (1997) has referred about the immunodiagnostic potential of *E. granulosus* adult-worm antigens in human cystic echinococcosis with an 87 % sensitivity. Antigen purification used to result in a lack of sensitivity while drawback of the highly sensitive antigen utilization is associated with the enhanced risk of cross-reactions. Poretti *et al.* (1999) reported up to 6 % cross-reactions in tumour patients and patients with other parasitic diseases. Based on analysis of our patient set it can be seen that every patient showed an individual immune response. The most important factor implying a diagnosis success was shown to be the localisation and character of an outer cyst layer. In any case it is necessary to proceed as follows: In case of suspected infection it is necessary to perform the serological analyses along with the clinical image techniques such as magnetic resonance, CT and USG. It is also necessary to use at least two different antigens in pre-screening ELISA (hydatid fluid of *E. granulosus* and somatic antigen of *E. multilocularis*). For differential diagnosis, findings should be confirmed by Western blot.

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