

Native and recombinant antigens in the immunodiagnosis of human cystic echinococcosis

ELENA ORTONA¹*, RACHELE RIGANÒ¹*, PAOLA MARGUTTI¹, SERGIO NOTARGIACOMO¹, SALVATORE IOPPOLO², SERGIO VACCARI¹, STEFANO BARCA¹, BRIGITTA BUTTARI¹, ELISABETTA PROFUMO¹, ANTONELLA TEGGI³ & ALESSANDRA SIRACUSANO¹

¹Laboratorio di Immunologia, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, ²Istituto di Tecnologie Biomediche, CNR, Roma and ³Dipartimento di Malattie Infettive e Tropicali, Università degli Studi 'La Sapienza', Viale Regina Elena 330, 00161 Roma, Italy

SUMMARY

To evaluate the diagnostic sensitivity and specificity of immunoelectrophoresis (IEP), indirect haemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA) and immunoblotting (IB), we compared their ability in detecting IgG antibodies to a hydatid fluid fraction (HFF) and to native and recombinant antigen B of *Echinococcus granulosus*. We tested sera from patients who had cystic echinococcosis (CE) grouped according to their type of cysts ($n = 204$), from patients with other parasitic diseases ($n = 21$), lung or liver carcinomas ($n = 6$) or serous cysts ($n = 26$) and from healthy controls ($n = 90$). HFF-IB gave the highest sensitivity (80%) followed by ELISA (72%), IHA (54%) and IEP (31%), respectively. The diagnostic sensitivity significantly ($P < 0.01$) decreased as cysts matured from type I-II to type VII. Recombinant and native antigen B-IB yielded similar sensitivity (74%). A large number of clinically or surgically confirmed CE patients (20%) resulted negative. In these patients' sera, IB to assess the usefulness of the recombinant *E. granulosus* elongation factor-1 β / δ in detecting IgE antibodies yielded 33% of positivity. Our findings underline the need to standardize techniques and antigenic preparations and to improve the performance of immunodiagnosis by characterizing new antigens and detecting distinct immunoglobulin classes.

Keywords *E. granulosus*, immunodiagnosis, seronegativity, IgE, elongation factor-1 β / δ

INTRODUCTION

Human cystic echinococcosis (CE), caused by *Echinococcus granulosus*, is an important public health problem in many regions of the world, both in areas where the disease is endemic, and in regions where the disease is emerging or re-emerging (Schantz *et al.* 1995, Craig *et al.* 1996). Early diagnosis is important because prompt intervention can reduce morbidity. Owing to the variable signs and symptoms of the disease, the clinical diagnosis of CE is difficult and requires physical examination, diagnostic imaging and serological techniques (Lightowers & Gottstein 1995). Immunodiagnosis is useful not only for the primary diagnosis but also for the follow up of patients after surgical or pharmacological treatment, or both. Immunodiagnostic techniques include initial screening tests, to identify crude antigens, such as latex agglutination, double diffusion, indirect haemoagglutination, and enzyme-linked immunosorbent assay, followed by confirmatory tests to identify specific antigens, for example arc-5 immunoelectrophoresis and immunoblotting (Lightowers & Gottstein 1995). The choice of a serodiagnostic technique depends primarily on its sensitivity and specificity. Despite the development of sensitive techniques, such as immunoblotting, the immunodiagnosis of CE remains a complex task. The first problem is that most conventional tests give a high percentage of false negative results (up to 25%). Other problems include the presence of false positive patients and the lack of standardization responsible for the discrepant results reported by the various laboratories.

The parasitic proteins that have major immunodiagnostic value in detecting *E. granulosus* are antigen 5 and antigen B. Antigen 5 has a 38 kDa component with phosphorylcholine epitopes that may be responsible for a large proportion of cross-reactions (Shepherd & McManus 1987, Lightowers *et al.* 1989). Although some immunological evidence suggests that antigen B also has a high diagnostic value, its

Correspondence: Alessandra Siracusano

Received: 18 January 2000

Accepted for publication: 15 June 2000

* Both authors contributed equally to this paper.

specificity remains controversial and it has limited use as an immunodiagnostic reagent (Shepherd *et al.* 1991, Rogan *et al.* 1991, Leggatt *et al.* 1992, Ioppolo *et al.* 1996). Few studies have assessed the diagnostic value of recombinant antigen 5 and antigen B (Facon *et al.* 1991, Frosch *et al.* 1994, Fernandez *et al.* 1996). Current research aims to investigate synthetic peptides derived from sequences of antigen 5 and antigen B (Leggatt & McManus 1994, Barbieri *et al.* 1998) and to isolate recombinant antigens that hold promise as new immunodiagnostic tools (Muhlschlegel *et al.* 1993, Ersfeld & Craig 1995, Martin *et al.* 1996, Colebrook & Lightowlers 1997). We have recently isolated an elongation factor-1 β/δ from a cDNA library of *E. granulosus* and have investigated its role in the host–parasite relationship. In immunoblotting analysis this protein showed immunological reactivity with sera from CE patients, in particular with IgE antibodies, yielding 52% sensitivity and 95% specificity (Margutti *et al.* 1999).

The study was designed to compare the sensitivity and specificity of four conventional immunodiagnostic techniques: immunoelectrophoresis (IEP), indirect haemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA) and immunoblotting (IB) to detect IgG antibodies to a hydatid fluid fraction (HFF) and to native and recombinant antigen B of *E. granulosus* in sera from patients who had CE, other parasitic diseases, lung or liver carcinomas or serous cysts and from healthy controls. We also analysed the serological reactivity of CE patients grouped according to their type of hydatid cysts. Finally, to improve the sensitivity of immunodiagnosis, we investigated whether the *E. granulosus* elongation factor-1 β/δ (EgEF-1 β/δ) recombinant antigen could be useful in detecting IgE antibodies in patients who tested seronegative in all four techniques.

MATERIALS AND METHODS

Antigens

Native antigens

Sheep hydatid fluid was collected from fertile cysts, clarified by centrifugation at 10 000 *g* for 60 min at 4 °C, five-fold concentrated in an Amicon ultra filtration cell with a YM2 membrane (Amicon Corp., Danvers, MA, USA) and kept at –20 °C for subsequent use. Two antigen preparations were used: an HFF, rich in antigen 5 and antigen B, obtained from crude sheep hydatid fluid by precipitation at low ionic strength (0.005 M acetate buffer, pH 5) according to Oriol *et al.* (1971); and native antigen B (AgB) eluted as previously described by Ioppolo *et al.* (1996).

Recombinant antigens

Two *E. granulosus* antigens were used: antigen B and the elongation factor-1 β/δ . Antigen B was obtained by polymerase

chain reaction (PCR) amplification of *E. granulosus* genomic DNA using primers derived from the antigen B sequence (Frosch *et al.* 1994). The following oligonucleotides with restriction sites were used: 5' primer (*Bam*H1 restriction site) 5'-ACGGGATCCATGCTTCTCG CTCTGGCTC-3'; 3' primer (*Eco*R1 restriction site) 5'-ACG CTTAAGGTAGATGGTTTATTGAGCA-3'. After PCR, the fragments were run in 2% agarose gel, purified with a Qiaex kit (Qiagen, GmbH, Germany) as instructed by the manufacturer, digested with restriction enzymes and cloned in GST pGEX vector (Amersham Pharmacia Biotech, Uppsala, Sweden). The GST fusion protein was expressed and purified by glutathione Sepharose 4B (Amersham Pharmacia Biotech) using the batch method following the manufacturer's instruction. The EgEF-1 β/δ was obtained as previously described from a cDNA library of *E. granulosus* (Margutti *et al.* 1999).

Blood samples

Blood samples were obtained from 204 CE patients recruited at the Department of Infectious and Tropical Diseases, University of Rome 'La Sapienza' (156 with cysts in the liver, 27 with cysts in the lungs, 15 with cysts in liver and lungs, five with cysts in bones and one with cysts in the nervous central system), 26 patients with serous hepatic cysts, 21 patients with other parasitic diseases (nine with schistosomiasis, five with trichinellosis, two with filariasis and five with *Taenia solium* cysticercosis), six patients with liver or lung carcinomas and 90 healthy donors. The patients with CE, serous hepatic cysts, liver or lung carcinomas and cysticercosis, attended the outpatient clinic to confirm diagnosis of CE based on clinical and imaging findings. In patients resulted serologically negative, surgery confirmed the diagnosis of CE. In addition, sera from patients with schistosomiasis, trichinellosis, filariasis and healthy donors were included as controls. Hydatid cysts were classified into seven different types according to Caremani *et al.* (1996). In brief, type I are simple cysts, type II are multiple cysts, type III are cysts with detachment of the wall, type IV are cysts with a mixed pattern, type V are heterogeneous cysts, type VI are hyperechoic cysts and type VII are calcified cysts. Sixty-four out of the 204 CE patients had type I cysts, 76 type II cysts, 36 type IV–V cysts and 28 type VII cysts. All procedures were approved by the local Ethical Committee and all subjects gave their informed consent to participate in the study. After collection, the sera were divided into aliquots and kept at –20 °C until use.

Serological tests

For IEP, HFF antigen (2 mg/ml) was run in a 1% agarose gel buffered with 0.05 M Veronal (pH 8.6) as previously

described by Bombardieri *et al.* (1974). IHA was tested as described by Bombardieri *et al.* (1974) in 96-well disposable microtitre plates (Greiner, Labour-Technik, Nürtingen, Germany). In brief, sheep blood cells were activated with tannic acid and then coated with 2 mg/ml of the HFF. A serum sample was considered positive when its agglutination ability remained in dilutions higher than 1:160. Patients' sera were tested by ELISA using either the HFF (2 µg/well) as described by Iacona *et al.* (1980), or native AgB (5 µg/well) as described by Ioppolo *et al.* (1996). Human sera were diluted 1:1000 and a goat antihuman IgG horseradish peroxidase conjugate (Bio-Rad, Richmond, CA, USA) was used as second serum. *O*-phenylenediamine dihydrochloride (Sigma Chemical Co., St Louis, MO, USA) was used as substrate and absorbance was measured at 495 nm. Means + 2 SD of the absorbance readings of the 90 healthy donors were considered as cut-off for positive reactions. Patients' sera were tested by IB as previously described by Siracusano *et al.* (1991). HFF, native or recombinant AgB and EgEF-1β/δ were run in SDS-PAGE in a 10% gel under nonreducing conditions. The antigenic subunits at 65 and 55 kDa correspond to antigen 5, the subunit at 8/12 kDa corresponds to antigen B. When EgEF-1β/δ was used as antigen, human sera were diluted 1:10 for IgE detection (Margutti *et al.* 1999) and a goat antihuman IgE peroxidase labelled serum (Cappel, Cochranville, PA, USA) was used as the second antibody. Two monoclonal antibodies, E5 specific for antigen 5 and A11B1 specific for antigen B, previously prepared in our laboratory, were used as controls and a goat antimouse IgG peroxidase labelled

serum (Bio-Rad) was used as second serum (Di Felice *et al.* 1986, Ortona *et al.* 1995).

Protein measurement

Protein concentrations were estimated using the Bio-Rad Bradford protein assay kit (Bio-Rad) and bovine plasma gamma globulin as a standard.

Statistical analysis

Sensitivity, specificity, positive and negative predictive values were calculated from a 2×2 table. Fisher's test was used for comparing sensitivity among patients grouped according to the type of hydatid cyst. $P \leq 0.01$ was considered statistically significant.

RESULTS

Serological reactivity to the antigen 5 and B-rich hydatid fluid fraction

IB showed positive reactions to antigen 5 alone (55–65 kDa subunits) or to antigen 5 and antigen B (8/12 kDa subunit) in 164 of the 204 sera from CE patients (80%). The other immunodiagnostic tests yielded lower sensitivity (ELISA, 72%; IHA, 54%; and IEP, 31%) (Table 1). Also in the five sera from patients with cysticercosis, IB detected more positive reactions than ELISA (80% versus 20%). One of the nine patients with schistosomiasis (11%) was

Table 1 Immunoelectrophoresis (IEP), indirect haemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA) and immunoblotting (IB) serological reactivity to hydatid fluid fraction (HFF) in sera from patients with CE, with other parasitic and non parasitic diseases and from healthy donors

Serum samples	No. sera	No. of positive samples (%)			
		IEP	IHA	ELISA	IB
Patients					
Cystic echinococcosis	204	63 (31)	110 (54)	145 (72)	164 (80)
<i>Taenia solium</i> cysticercosis	5	—	—	1 (20)	4 (80)
Schistosomiasis	9	—	—	1 (11)	—
Trichinellosis	5	—	—	—	—
Filariasis	2	—	—	—	—
Liver/lung carcinomas	6	—	—	—	—
Serous cysts	26	—	—	2 (8)	2 (8)
Healthy donors	90	—	—	—	—
Sensitivity (%)		31	54	72	80
Specificity (%)		100	100	97	96
Positive predictive value (%)		100	100	97	96
Negative predictive value (%)		50	60	70	77

Serum samples	No. sera	No. of positive samples		
		ELISA	IB	
		nAgB	nAgB	rAgB
Patients				
Cystic echinococcosis	204	150	135	147
<i>Taenia solium</i> cysticercosis	5	—	—	—
Schistosomiasis	9	—	—	Not tested*
Trichinellosis	5	—	—	—
Filariasis	2	—	—	—
Liver/lung carcinomas	6	—	—	—
Serous cysts	26	—	—	—
Healthy donors	90	—	—	—
Sensitivity (%)		74	66	72
Specificity (%)		100	100	100
Positive predictive value (%)		100	100	100
Negative predictive value (%)		73	67	70

* The presence of *S. mansoni* GST in the recombinant protein prevented us to evaluate the reactivity of sera from patients affected by schistosomiasis.

ELISA-positive only. Two of the 26 patients with serous cysts (8%) were ELISA- and IB-positive; the nature of serous cysts was confirmed by parasitological and histological techniques. These patients had history of oxyuriasis during childhood. All techniques correctly yielded negative results in sera from patients with trichinellosis and filariasis or with lung or liver carcinomas and in sera from healthy blood donors. These data yielded 100% specificity in IEP and IHA, 97% in ELISA and 96% in IB. A total 64 of all CE patients' sera (31%) were positive in all immunodiagnostic tests, 46 (23%) in IHA, ELISA and IB, 35 (17%) in ELISA and IB and 19 (9%) in IB alone. In an unexpectedly large

number of patients with clinically or surgically confirmed CE ($n = 40$, 20%), all the serodiagnostic techniques yielded negative results.

Serological reactivity to native and recombinant antigen B

The serological reactivity of sera to native AgB was tested by ELISA and IB. Recombinant AgB was tested in IB alone because it contained bacterial contaminants. Native AgB-ELISA, native AgB-IB and recombinant AgB-IB detected almost the same percentage of positive reactions (74% and 72%, respectively) (Table 2). None of the sera from patients with cysticercosis, schistosomiasis, trichinellosis, filariasis, lung or liver carcinomas, serous cysts and from healthy blood donors cross-reacted with native or recombinant AgB.

Serological reactivity in patients divided according to the type of cyst

All serodiagnostic techniques yielded the highest percentage of positive reactions in subjects with simple (type I) or multiple cysts (type II). As cysts matured from type I–II to type VII, the sensitivity significantly decreased ($P < 0.01$) (Figure 1).

When we analysed the distribution of cyst types in the 40 IEP-, IHA-, IB-, ELISA-negative patients, we found that calcified cysts accounted for the highest percentage (type VII cysts, 62%; type IV–V and II, 15%; and type I, 8%).

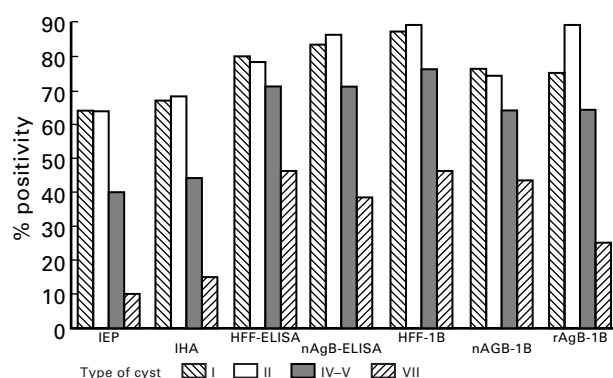


Figure 1 Pattern of positive immunoelectrophoresis (IEP), indirect haemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA) and immunoblotting (IB) reactions in the 204 CE patients divided according to the type of cysts.

Serological reactivity to recombinant *E. granulosus* elongation factor-1 β / δ

IB to detect IgE antibodies against recombinant EgEF-1 β / δ and HFF showed that 13 of the 40 seronegative patients' sera (33%) reacted to EgEF-1 β / δ . Six of these 13 patients (46%) had calcified cysts (type VII), three (23%) had type IV-V cysts, three (23%) had type II cysts and only one (8%) had type I cysts. None of the seronegative patients had IgE to HFF.

DISCUSSION

Providing more sensitive and specific serologic tests for CE is still a pressing research aim owing to the increasing number of seronegative patients and the growing request for immunodiagnosis in patients coming from countries with endemic cross-reactive parasitic diseases.

This study confirms IB, followed by ELISA, as the most sensitive of the currently available immunodiagnostic techniques in detecting antibodies to *E. granulosus* antigens (Siracusano *et al.* 1991). Hence, we recommend using IB in the diagnostic work-up of patients with suspected CE, reserving ELISA, the less sensitive test to monitor antibody titres during follow-up. Although the low sensitivity of IHA and IEP limits their usefulness in serodiagnosis, both tests are still in use, IHA for semiquantitative and IEP for qualitative analysis of specific antibodies.

The reported diagnostic sensitivity of IB using hydatid fluid ranges widely, from 50–91% (Lightowers & Gottstein 1995, Porretti *et al.* 1999). In earlier studies, we found that IB achieved 90% sensitivity with HFF and 80% with native AgB (Siracusano *et al.* 1991, Ioppolo *et al.* 1996). The lower sensitivity of HFF-IB (80%) and native AgB-IB found in this study might depend on differences in antigen preparation, or on clinical features of the patients, including the number, size, location, integrity and morphology of the cysts. Although the antigens for our studies come exclusively from hydatid fluid obtained from sheep liver, we cannot exclude qualitative and quantitative antigenic variations related for example to parasite strains, or host characteristics. As crude antigen, other laboratories use hydatid fluid from cattle, horses, or camels originating from various geographical areas (Shambesh *et al.* 1995, Porretti *et al.* 1999). Antigenic preparations for use in immunodiagnostic techniques therefore need to be qualitatively and quantitatively standardized, for example by a panel of recombinant antigens or synthetic peptides (Leggatt & McManus 1994, Barbieri *et al.* 1998). The serological sensitivity of the recombinant AgB-IB for detecting IgG in this study (72%) approaches that found by McVie *et al.* (1997) who used dot-ELISA (74%).

The discrepant sensitivity reported by the various laboratories could also arise from differences in the clinical features of the host or in the time of serum sampling (pre- or postsurgical or pharmacological treatment). The sensitivity of all techniques in this study significantly decreased as the cyst matured from type I–II to type VII and, in particular, IHA and IEP invariably had low sensitivity in patients with calcified cysts (type VII). Interestingly, in patients with calcified cysts, IB achieved higher sensitivity with native AgB than with recombinant AgB, probably due to the presence of immunodominant glycidic epitopes or to the oligomeric structure of native AgB (Gonzales *et al.* 1996). Further studies are necessary to clarify the role of this antigen in distinct stages of the disease. The diagnostic value of the available serological techniques as primary screening tests or as confirmation tests must therefore be assessed in a homogeneous population of patients. In addition, studies designed to assess the diagnostic performance of the various available antigens should use the same panel of antisera, as Lightowers & Gottstein (1995) have already noted.

In a recent study investigating a new *E. granulosus* antigen, EgEF-1 β / δ , we found that CE patients identified as having IgE against EgEF-1 β / δ were predominantly those with calcified cysts (type VII) (Margutti *et al.* 1999). One explanation could be that substantial amounts of EgEF-1 β / δ are released in the hydatid fluid during or after the degeneration of protoscoleces. In this study, the high percentage (62%) of seronegative patients with calcified cysts (type VII) prompted us to investigate in these subjects the IgE reactivity against the EgEF-1 β / δ in seronegative subjects. A high percentage of sera (33%) tested positive for IgE to EgEF-1 β / δ and negative for IgE to HFF. Hence, we conclude that identifying IgE specific for EgEF-1 β / δ could improve the diagnostic sensitivity of IB. In our study, the sensitivity of IB increased from 80% to 87%. Whether this antigen has a place in the immunosurveillance of CE as a marker of parasite degeneration remains a question for further research. Previously, we suggested that immunological regulatory or effector mechanisms differ in seropositive and seronegative patients (Riganò *et al.* 1998). The downregulation of Th2 cytokines found in our previous study could explain why seronegative patients are unable or less able to produce antibodies in response to parasites. The detection of IgE in our seronegative patients demonstrates that these patients can produce immunoglobulins. Another question arising from these findings is whether the immunodiagnosis of CE might be improved by using various immunoglobulin classes specific for distinct antigens.

Although most of the immunodiagnostic techniques we studied yielded acceptable sensitivity in detecting IgG against HFF, this fraction cross-reacted with sera from

patients with cysticercosis and schistosomiasis. The specificity of antigen B remains controversial. Ito *et al.* (1999) found that 92% of CE sera, as well as 79% of alveolar echinococcosis sera, reacted with the 8 kDa subunit of antigen B, and showed that this antigen is not species-specific for *E. granulosus* but is genus-specific for *Echinococcus*. Leggatt *et al.* (1992) reported its cross-reactivity with a small percentage of patients with cysticercosis. In accordance with Ito *et al.* (1999), we found that antigen B did not cross-react with sera from patients with other parasitic diseases. Overall, our data support the results of Porretti *et al.* (1999), who reported that the specificity of immunodiagnosis can be improved by using IB to detect antibodies to antigen B, in particular the 8 kDa subunit.

Notably, the use of antigen B in immunodiagnosis increases specificity but decreases sensitivity. In addition, the more widespread use of imaging techniques has increased requests for serodiagnosis in asymptomatic patients with young (early stage) or calcified (late stage) cysts, frequently low responders or seronegative patients. Hence, false seronegativity is nowadays the most pressing problem in CE immunodiagnosis.

In conclusion, current strategies for the immunodiagnosis of CE need reappraisal. Improving their diagnostic yield means undertaking studies to standardize techniques and antigen preparations; to characterize new antigens; to produce recombinant antigens; and to detect distinct immunoglobulin classes.

ACKNOWLEDGEMENTS

We are in debt to Dr E. Martini, Dr R. Buzzico, Dr R. Pezzato, Dr M. Borchia for the kind gift of the hydatid fluid and to Mr N. Di Carlo for his valuable cooperation.

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