

SERODIAGNOSIS OF ALVEOLAR ECHINOCOCCOSIS : DETECTION OF ANTIBODY AGAINST EM18 IN PATIENTS AND RODENTS

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Abstract. An international collaborative study on echinococcosis has been carried out for the establishment of a simple means for differential serodiagnosis of alveolar echinococcosis (AE) from other parasitic diseases including cystic echinococcosis (CE). The main candidate epitope is Em18 (previously undescribed epitope of a low molecular weight protein of 18.5 kDa). Evaluation of the usefulness of Em18 is introduced in this review paper. Serum samples showing antibody response against Em18 are exclusively from AE. The predominant IgG subclass recognizing Em18 is IgG4 or IgG1 or IgG4 + IgG1 but never IgG2. There are good correlations between (1) the antibody response against Em18 and the presence of active lesions and (2) the antibody response against Em18 and the Em2-ELISA values. Em18 is, therefore, expected to be reasonably reliable and useful for differentiation of active AE from inactive AE. A new ELISA system using a partially purified Em18 enriched fraction (PP-Em18/16-ELISA) has been evaluated for serodiagnosis of AE compared with Em2plus-ELISA. A total of 194 serum samples were examined: 127 sera from AE (79) and CE (48) in China where both AE and CE are endemic, 21 sera from CE in Australia where CE only exists, 28 sera from cysticercosis (21), paragonimiasis (5) or sparganosis (2) in Korea where no indigenous AE nor CE exists and 11 normal sera. Antibody levels by PP-Em18/16-ELISA were much higher in AE than in CE and it was also true for commercially available Em2plus-ELISA. Some of CE from China showed exceptionally higher levels of antibody in comparison with those of CE from Australia. It is suggested that these strongly positive cases of CE from China may have been exposed to both species of *Echinococcus*. Although most of sera from paragonimiasis showed high antibody levels by Em2plus-ELISA, they were negative by PP-Em18/16-ELISA. Therefore, PP-Em18/16-ELISA is expected to be more reliable for differentiation of AE from CE and others especially in Asian countries where paragonimiasis is still not rare. Antibody responses in rodents naturally infected with *E. multilocularis*: Serum samples from the wild vole, *Clethrionomys rufocanus bedfordiae*, infected with *E. multilocularis* showed similar antibody responses as in AE patients, whereas those from Norway rats, *Rattus norvegicus*, showed almost none. The latter rodents were simultaneously infected with *Taenia taeniaeformis* but showed no antibody response against *T. taeniaeformis* either. Therefore, we speculate that Norway rats may only be infected with *E. multilocularis* under some immunosuppressed conditions or genetic unresponsiveness. It is stressed that Em18 is highly specific to *E. multilocularis*, and antibody response against Em18 is reasonably reliable for differentiation of AE from other helminthic infections by Western blot and ELISA in humans and may be useful for detection of domestic animals contaminated with *E. multilocularis* in the endemic area.

INTRODUCTION

Echinococcosis, either alveolar (AE) or cystic (CE), is one of the most pathogenic helminthic infections of humans. AE, often misdiagnosed as hepatic cancer (Kasai *et al*, 1989), is caused by the larval stage of the fox tapeworm, *Echinococcus multilocularis*, whereas CE is by that of the dog tapeworm, *E. granulosus*. Recent epidemiological study has revealed that AE is widely spreading in the northern hemisphere other than tropical or subtropical areas (Schantz *et al*, 1991; Craig *et al*, 1991, 1992)

including Hokkaido, Japan (Suzuki *et al*, 1993). However CE is cosmopolitan. Due to the geographical distribution of AE and CE, both AE and CE are very common in some parts of Eurasia (Craig *et al*, 1992) and such areas endemic for both are speculated to be expanding in Eurasia year by year. Furthermore, *E. multilocularis* is becoming enzootic in an increasing area of northcentral North America (Storandt and Kazacos 1993; Schantz, 1993). Therefore, AE is now recognized as an important public health problem.

Due to the fact that AE and CE are caused by different species of genus *Echinococcus* and the clinical manifestations including morbidity and mortality critically differ between them, the establishment of improved methods for early detection of AE patients with active lesions is a critical need, since in many cases, patients are diagnosed after AE has advanced to the point that lesions are nondetectable and the outcome is fatal (Schantz *et al*, 1982).

Serologic screening with an enzyme-linked immunosorbent assay (ELISA), using a partially purified antigenic component of *E. multilocularis* (Em2) has been used widely in endemic areas for this purpose (Gottstein *et al*, 1983, reviewed by Gottstein, 1992). However, it is now known that seropositive results in the Em2-ELISA do not always correlate with the status of disease dynamics, since patients with inactive, calcified lesions after spontaneous death of the parasite may also be Em2-ELISA positive (Rausch *et al*, 1987; Ito *et al*, 1995a). This might be mainly due to the fact that Em2 is not protein but carbohydrate (Deplazes *et al*, 1991) and locating exclusively on the laminated layer (Sturm *et al*, 1995, reviewed by Gottstein and Felleisen, 1995).

Recently, we identified two protein epitopes of this parasite, designated Em18 and Em16, which appeared to be good markers for the presence of lesions of AE (Ito *et al*, 1993a, b). In the present paper, I introduce recent international collaborative work for evaluation of these two new potential markers for differential serodiagnosis of AE from CE and other helminthic diseases (Ito *et al*, 1995a, b, c; Wen *et al*, 1995).

MATERIALS AND METHODS

Sera: Nineteen AE serum samples from Japan and 39 CE from Australia were examined initially to determine Em18 and Em16 (Ito *et al*, 1993a). Thirty-three serum samples, either AE or CE or normal, from China were examined for the second study to differentiate AE from CE or normal, based on antibody responses against Em18 and Em16 (Ito *et al*, 1993b). Thirty-six unknown serum samples including at least some AE and one confirmed double infection of AE + CE (Wen *et al*, 1992), collected from clinical cases and confirmed in Urumqi, Xinjiang, China were examined for the third study to differentiate AE + CE from AE or CE. Seven CE, five AE, one

AE + CE (double infection) from Urumqi and three CE and 22 AE from Chongqing, China, were examined for IgG subclass responses against Em18 and Em16 (Ito *et al*, 1995b). Fifteen Alaskan AE serum samples including inactive cases, suspected to be AE clinically and confirmed pathologically at the Centers for Disease Control and Prevention (CDC) were examined for the fourth study to differentiate active from inactive AE (Ito *et al*, 1995a). Additional serum samples collected from clinical patients and confirmed in Chongqing (79 AE and 48 CE), Seoul (28 cysticercosis, 2 sparganosis, 5 paragonimiasis) and Melbourne (21 CE) were examined for Em2plus-ELISA and our ELISA using partially purified Em18/Em16 enriched fraction (PP-Em18/16-ELISA) (Ito *et al*, 1995c). Further evaluation was carried out using 34 paragonimiasis skrjabinii, 30 clonorchiasis, 50 schistosomiasis, 33 cysticercosis from China, and 20 cysticercosis and 30 paragonimiasis westermanii, 10 sparganosis and 30 hepatic cancer from Korea (Ito *et al*, 1995c). An additional 40 and 81 serum samples for a blind test were from the USA and Switzerland, respectively (Ito *et al*, unpublished).

Serologic analysis: SDS-PAGE and transblotting were carried out using commercially available precast 18 % isocratic or 4 - 20 % gradient gels (01-102, -106, -022, -026, SDS-PAGE Mini, Tefco, Tokyo, Japan). Western blot analysis was carried out using a 1 : 50 or 1 : 100 dilution of serum samples tested at least twice. Antibody responses against Em18 and/or Em16 were assessed using peroxidase-conjugated anti-human IgG (Cappel, USA) and monoclonal antibodies (MAbs) against human IgG subclasses (G1, G2, G3, G4)(Zymed, UK) at a 1: 500 dilution. For detection of antibody responses against Em18 and Em16, pooled sera from Japanese and Chinese AE and a MAb against Em16 were used as standard positive controls, respectively (Ito *et al*, 1993a, b; Ito *et al*, 1995a, b, c) The Em2-ELISA values were analyzed at CDC (Lanier *et al*, 1987). Em2plus-ELISA, purchased from Bordier Affinity Products, Switzerland, and ELISA using partially purified Em18/Em16 enriched fraction (PP-Em18/16-ELISA) were carried out using a 1: 200 dilution of serum samples from Chongqing, Seoul and Melbourne (Ito *et al*, 1995c). For detection of antibody responses in rats, peroxidase-conjugated anti-rat Ig G (Cappel, USA) in 1: 1,000 dilution was used, whereas for detection of those in wild rodent, *Clethrionomys rufocanus bedfordiae* (Crb), biotin-labeled anti-Crb-IgG was produced in rabbits by ourselves (Ito *et al*, 1994).

RESULTS AND DISCUSSION

Differentiation of AE from other helminthic diseases (Ito *et al.*, 1993a,b; Ito *et al.*, 1995a).

The purpose of this study was to identify species-specific protein components of *Echinococcus multilocularis* by Western blot analysis and establish a simple and highly sensitive method for differential serodiagnosis of AE. For this study, we used Japanese AE and Australian CE and other parasitic diseases, at first. Two, previously undescribed components, designated Em18 and Em16 due to their molecular weights, appeared to be unique to AE (Ito *et al.*, 1993a). Based on this observation, we examined 33 Chinese sera as a blind test (Ito *et al.*, 1993b). The summary of antibody responses against Em18 and/or Em16 in the 33 Chinese serum samples are shown in Table 1. Sera antibody positive against Em18 were from all 15 AE

and two of 14 CE. One AE showing the most weak response against Em18 was from a patient presumably cured after albendazole treatment. It was doubted that the 2CE had been exposed to both, since there was one paper reporting double infections case in China (Wen *et al.*, 1992).

The third study was carried out using 36 serum samples from Urumqi, China. Antibody responses against Em18 and/or Em16 and confirmed clinical of 36 serum samples from Urumqi are summarized in Table 2. All AE cases, except one (1/6) who exhibited calcified lesion only, showed antibody responses against Em18 and Em16. Therefore it seemed probable that AE with calcified lesion showed weak or no antibody responses against Em18 or Em16. We expect that follow up studies of antibody responses against Em18 might be useful for monitoring prognosis.

Table 1

Summary of antibody responses against Em18 and/or Em16 in 33 Chinese serum samples (modified from Ito *et al.*, 1993b).

No. of samples	Antibody responses				Active AE
	Em18 + Em16	Em18 only	Em16 only	negative	
All (33)	15/33	5/33	1/33	12/33	21/33
15 AE	11/15	4/15	0/15	0/15	15/15
14 CE	4 ^a /14	1 ^b /14	1/14	8/14	5 ^c /14
4 normal	0/4	0/4	0/4	4/4	0/4

a : three were uncertain. b : uncertain. c : one positive against Em16 is not included, since there is no AE cases showing antibody response against Em16 exclusively (Ito *et al.*, 1995b, c).

Table 2

Antibody responses against Em18 and/or Em16 and confirmed clinical diagnoses of 36 Chinese cases (from Ito *et al.*, 1995b).

36 cases	Antibody responses			
	Em18 + Em16	Em18 only	Em16 only	negative
6 AE	5	0	0	1 ^a
1 AE + CE	1	0	0	0
22 CE	0	1 ^b	6	15
7 normal	0	0	0	7

a : almost calcified case. b : the first CE case showing clear response (1/36)(see Ito *et al.*, 1993b).

The single case of double infection showed antibody responses against Em18 and Em16 similar to the typical AE. In contrast, most of CE (15/22) did not show any responses against Em18 or Em16. However, six of the seven showed antibody response against Em16 and not against Em18. This picture was also observed when we examined CE in Sichuan, China (Table 1, Ito *et al.*, 1993b). Therefore, it seemed probable to speculate that antibody response exclusively against Em16 might be specific or unique to CE. There were only two cases showed antibody responses against Em18 in 36 Chinese CE: one was against Em18 and Em16 (Ito *et al.*, 1993b) and the other was against Em18 only (Ito *et al.*, 1995b). These cases were misdiagnosed as AE.

Wen and Craig (1994) confirmed that Em18 was species specific for *E. multilocularis*. They also observed that one of 81 Chinese CE recognized Em16 and a very small number of Chinese CE patient sera exclusively cross-reacted with Em18. Although there is only one confirmed double infection case (Wen *et al.*, 1992), we speculate that such double infection may not be rare. In order to evaluate this speculation, we compared antibody responses against Em18 and Em16 in CE sera from China where AE and CE are endemic and from Uruguay and Libya where there is no AE. Thus far, there is no evidence of CE from Uruguay and Libya which show antibody responses against Em18 and Em16 or against Em18 (Wen *et al.*, 1995). These results support the speculation. However, we should test more cases for conclusive evidence.

Differentiation of active AE from inactive AE (Ito *et al.*, 1995a).

Fifteen Alaskan patients with either active or

inactive lesions of AE, previously confirmed clinically, pathologically, and serologically by the Em2-ELISA, were used for a blind test by Western blot analysis. Ten and five cases were considered to be active and inactive cases, respectively by antibody response against Em18 Western blot. One of the 10 cases classified serologically as active was judged to be inactive based on clinical and pathologic criteria; the patient had a recognizable parasite lesion, and followed short-term treatment with albendazole, a biopsy of the liver showed a degenerated lesion that did not grow in rodents. However, the absence of growth of the parasite in rodents does not always mean conclusive evidence of the parasite's death. So, this case should be classified as a "presumptive" inactive case. The five cases judged to be inactive due to the absence of response against Em18 included two confirmed inactive cases with calcified lesions at CDC and three active cases that showed the weakest values in the Em2-ELISA at CDC. Correlation between antibody responses against Em18 by Western blot analysis and Em2-ELISA and clinical status of the 15 Alaskan cases of AE is summarized in Table 3: one of the confirmed inactive cases which showed high value in Em2-ELISA was negative in Em18 Western blot analysis. In the most predominant IgG subclass responding to Em18 was IgG4. In general, there were good correlations between (1) the antibody response against Em18 and the presence of active lesions and (2) the antibody response against Em18 and the Em2-ELISA values. As the number of sera examined was not many, similar work will be necessary to evaluate the usefulness of Em18 as a candidate marker for differentiation of active from inactive AE.

Table 3

Correlation between antibody responses against Em18 (Western blot) and Em2 (ELISA) and clinical status of 15 Alaskan cases of AE (modified from Ito *et al.*, 1995a).

Clinical status confirmed at CDC	Antibody responses against	
	Em18 at Gifu	Em2 at CDC
Active (9 cases)	>+	13% ≤ 100%
Active (3 cases)	-	5%, 10%, NT
Presumptive inactive (1 case) ^a	+	25%
Confirmed inactive (2 cases)	-	86% ^b , 0%

a : inactive after one year of chemotherapy; b : The most interesting inactive case with high value in Em2-ELISA.

Differentiation of AE and CE from cysticercosis by Em16 (Ito *et al*, unpublished).

Although antibody responses against Em18 and Em16 are expected to be highly reliable new serodiagnostic markers for AE and CE and there is a report that there is no cross reaction against Em18 or Em16 using 37 cysticercosis serum samples (Wen and Craig, 1994). There were however some responses around Em18 or Em16 with some cysticercosis sera (Table 4, Ito *et al*, unpublished). Recent work has suggested that these cross reactions are not directed against these two epitopes, or at least not against Em18. The most clear cut evidence that Em16 is shared with *E. multilocularis* and *E. granulosus* but not with *Taenia solium* has been demonstrated by Western blot analysis using MoAb against Em16 (Ito *et al*, unpublished). Therefore, we expect to establish a new means for the differential serodiagnosis of AE and CE by new markers, Em18 and Em16. Purification of Em18 and Em16 and production of recombinant Em18 and Em16 are under study.

Table 4

Antibody responses against Em18 for differentiation of AE from 77 unknown serum samples from CDC and University of Zurich (Ito *et al*, unpublished).

	Antibody responses against Em18		
	+	+?	-
AE (42)	33	2(1 ^b)	7(2 ^a + 2 ^b)
CE (17)	1	4	12
Cysticercosis (8)	0(3 ^c)	0(4 ^c)	8(1 ^c)
Trematodioses (5)	0	0(1 ^c)	5(4 ^c)
Nematodioses (5)	0	0	5

37 and 40 samples from CDC and University of Zurich, respectively.

a : Two are from one patient before and after treatment.

b : confirmed inactive cases.

c : these showed some response around (but not against) Em18.

Em2plus-ELISA vs partially purified Em18/16-ELISA (Ito *et al*, 1995c)

Using additional sera from China (79 AE and 48CE), Australia (21 CE) and Korea (28 cysticercosis, 5 paragonimiasis and 2 sparganosis), we compared the

values between Em2plus-ELISA and our ELISA using partially purified Em18/Em16 enriched fraction (PP-Em18/16-ELISA) and analyzed correlation with Western blot Em18/Em16 figures (Tables 5, 6). PP-Em18/16 was prepared by isofocusing. In general, there were good correlations between Em2plus-ELISA and PP-Em18/16-ELISA and between these values and Western blot Em18/Em16 figures. When a negative cut-off value for the Em2plus-ELISA was determined using the weak-positive serum supplied with the kit, there were false positive reactions to paragonimiasis (3/5), sparganosis (1/2), CE (15/69) and cysticercosis (1/28). Based on the false positiveness of CE, the cut-off value in PP-Em18/16-ELISA was established. The false positive reactions disappeared except for CE that remained unchanged (16/69). Therefore, the reliability of serodiagnosis of AE by PP-Em18/16-ELISA was significantly better than Em2plus-ELISA. Some of CE in China showed extremely high values by Em2plus-ELISA and PP-Em18/16-ELISA, whereas those in Australia showed only low values. These might support our speculation that there might be more chances for the residents in the endemic area for both AE and CE to be infected with both species (Ito *et al*, 1993b; Wen *et al*, 1995). Further clinical follow-up studies are under progress in China (Ma *et al*, unpublished).

Detection of wild and/or domesticated animals infected with *E. multilocularis* (Ito *et al*, 1994; Ito *et al*, in preparation)

AE and CE are serious helminthic zoonoses. For prevention, control and surveillance of echinococcosis, it is very important to obtain information on the infection dynamics in the wild or domesticated, intermediate and definitive host animals. Recently, we succeeded in obtaining serum samples from the wild vole, *Clethrionomys rufocanus bedfordiae*, naturally infected with *E. multilocularis*. Their antibody responses appeared to be similar to human cases but different from those from mice infected intraperitoneally with protoscoleces (Table 7) (Ito *et al*, 1994). Although the wild vole is known as a suitable intermediate host for *E. multilocularis* in Japan, there are two cases of Norway rats naturally infected with this parasite. In order to evaluate the niche of the rat for completion of the life cycle of this parasite, antibody responses against (a) this parasite antigens and (b) *Taenia taeniaeformis* antigens were examined, since one of the two rats were infected with the two species

Table 5

Reliability of Em2plus-ELISA and PP-Em18/16-ELISA for differentiation of alveolar echinococcosis (AE) from other parasitic diseases including cystic echinococcosis (CE) (Ito *et al*, 1995c).

Sera from	Em2plus-ELISA			PP-Em18/16-ELISA		
	positive	negative	%	positive	negative	%
Chinese AE (79) ^a	63	16	79.7	72	7	91.1
Chinese CE (48) ^b	15	33	31.3	16	32	33.3
Australian CE (21)	2	19	9.5	0	15	0.0
Cysticercosis (28) ^c	1	27	3.6	0	28	0.0
Sparganosis (2) ^c	0	2	0.0	0	2	0.0
Paragonimiasis (5) ^c	3	2	60.0	0	5	0.0

No. of patients other than Chinese AE and CE; a : 79 samples from 24 patients; b : 48 samples from 34 patients; c : from South Korea.

Table 6

Sensitivity and specificity of three different serologic methods for differentiation of alveolar echinococcosis (AE) from cystic echinococcosis (CE) (Ito *et al*, 1995c).

No. of patients	Em2plus-ELISA		PP-Em18/16-ELISA		WB-Em18/Em16			
	positive	negative	positive	negative	+/+	+/-	-/+	-/-
Chinese AE (24)	21	3	24	0	24	0	0	0
Chinese CE (34)	10	24	13	21	9	0	4	21

Table 7

Antibody responses against Em18 and Em16 in wild voles, humans and mice and proliferation of protoscoleces (PS)(based on Ito *et al*, 1994; Ito *et al*, unpublished),

	Host animals			
	Norway rats	Wild voles	Humans	Mice
Antibody responses against Em18/Em16	-/-	+++ /+++	+++ /+++ ^a ++ /+ ^a + /- ^a	++ /+++ ^b + /++ ^b - /++ ^b
Infection with	eggs	eggs	eggs	PS
Proliferation of PS	very poor	extremely high	poor	high

a : see Ito *et al*, 1993a, b; b : highly variable among mouse strains (Ito *et al*, unpublished).

in the liver. There was almost no antibody responses against *E. multilocularis* nor *T. taeniaeformis* (Ito *et al.*, in preparation). Therefore, we speculate that the Norway rat was either under some immunodeficiency or genetic unresponsiveness as reported in *Hymenolepis citelli* (Wassom *et al.*, 1974, reviewed by Ito and Smyth, 1987). However, this finding might present a difficult problem in control of this parasitic infection.

There are many cases in pigs naturally infected with this parasite in Japan (Sakui *et al.*, 1984) and in Europe. Therefore, it would be interesting to know if we can detect antibody responses against this parasite or Em18. For further evaluation of the usefulness of antibody response against Em18 in humans and animals, it is important to analyse the infection dynamics from oral egg infection and the dynamics in antibody responses, especially against Em18.

These results described above appear to support the idea that Em18 may be reliable marker for serodiagnosis of alveolar echinococcosis not only in human but also in animals which may be served as the intermediate host. We expect to establish more reliable means for differential serodiagnosis of AE and CE when we can use purified Em18 or recombinant Em18.

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