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Journal of Clinical Microbiology (2002. Nov) 40(11):4161–4165.

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Evaluation of an Enzyme-Linked Immunosorbent Assay (ELISA) with Affinity-Purified Em18 and an ELISA with Recombinant Em18 for Differential Diagnosis of Alveolar Echinococcosis: Results of a Blind Test

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Received 3 May 2002/Returned for modification 9 July 2002/Accepted 30 July 2002

Alveolar echinococcosis (AE) is the most potentially lethal parasitic zoonosis of the nontropical areas in the northern hemisphere, where cystic echinococcosis (CE) is also endemic. Both AE and CE are highly endemic in China, and both serologic detection of echinococcosis, either AE or CE, and differentiation of AE from CE are crucial problems. Evaluation of Western blot analysis (WB) and enzyme-linked immunosorbent assay (ELISA) for the Em18 antigen, using affinity-purified and recombinant Em18, was carried out “blindly” using 60 human sera from patients diagnosed in France. The results were compared with those obtained using a commercially available *Echinococcus* WB immunoglobulin G (IgG) kit developed in France. The Em18 WB and *Echinococcus* WB IgG showed very similar results for detection of AE. Both affinity-purified Em18 or a recombinant Em18 WB and *Echinococcus* WB IgG seem useful for identification of AE, and the latter seems appropriate for both AE and CE, whereas affinity-purified Em18 ELISA and the newly developed recombinant Em18 ELISA appear to be suitable for detection of AE, especially for epidemiological surveys.

Alveolar echinococcosis (AE), caused by the metacestode of the fox tapeworm, *Echinococcus multilocularis*, is the most potentially lethal parasitic zoonosis of the nontropical areas in the northern hemisphere, where cystic echinococcosis (CE), caused by the metacestodes of the dog tapeworm, *E. granulosus*, is also endemic. For example, both AE and CE are highly endemic in China (8, 19) and serological detection of echinococcosis, either AE or CE, and differentiation of AE from CE are crucial problems, since the pathogenicity of these two types of echinococcosis and the treatment of patients with these diseases are critically different (14, 16).

In Japan and France, immunoblotting (Western blotting [WB]) assay systems have been developed for differentiation of AE from other diseases (4–7). The Asahikawa Medical College (AMC) group in Japan has focused on the detection of antibody response to the Em18 antigen (approximately 18 kDa) extracted from protoscoleces of *E. multilocularis* (4, 9, 15) and has tried to purify Em18, which shows a single band in WB, and to make it available for enzyme-linked immunosorbent assay (ELISA), using preparative isoelectric focusing (PIEF). Since purification of Em18 by PIEF takes longer and the yield is not as great, we have shifted to purification of Em18 by affinity chromatography (AffEm18) and production of a recombinant

Em18 (RecEm18) for WB and ELISA (7, 9, 10, 13, 18). *Echinococcus* WB immunoglobulin G (IgG) (EchWB IgG; LDBIO Diagnostics, Lyon, France), which has a high sensitivity for the detection of both AE and CE, is basically very similar to the AMC system since it also focuses on differentiation of AE and CE based on different banding patterns including antigen B (most predominant at 8 and 26 to 28 kDa), Em16, and Em18 in crude antigens. The merit of the latter system is that it detects both AE and CE on a single strip based on the difference in the banding patterns. In this paper, we report blind test results from AffEm18 and RecEm18 WB and AffEm18 and RecEm18 ELISA using 60 serum samples prepared in France for further evaluation of the usefulness of Em18 serology for identification of AE.

MATERIALS AND METHODS

Serology. AffEm18 was prepared as follows. Protoscoleces of *E. multilocularis* were homogenized in 0.1 M Tris-HCl (pH 7.5) containing 0.5 M NaCl and 1% NP-40. After centrifugation, the resulting supernatant was incubated at 37°C for 1.5 h and applied to an affinity resin column (Hi Trap NHS-activated HP; Amersham Pharmacia Biotech) coupled with anti-Em18 polyclonal antibody. After unbound proteins were washed off with 0.1 M Tris-HCl (pH 7.5) containing 0.15 M NaCl and 1% NP-40, Em18 was eluted with 0.1 M glycine-HCl (pH 2.6). The eluates were pooled and used for further experiments. For AffEm18 WB and AffEm18 ELISA, 2.5- μ g samples per mini sodium dodecyl sulfate-polyacrylamide gel (6 cm wide) (Tefco, Tokyo, Japan) and 250 ng per well (Maxisorp; Nunc, Copenhagen, Denmark) were used, respectively. A RecEm18 produced at AMC (9) was also used for this project. For RecEm18 WB and RecEm18 ELISA, 350 ng per mini sodium dodecyl sulfate-polyacrylamide gel and 50 ng per well were used, respectively. WB and ELISA were carried out as reported previously (5, 6,

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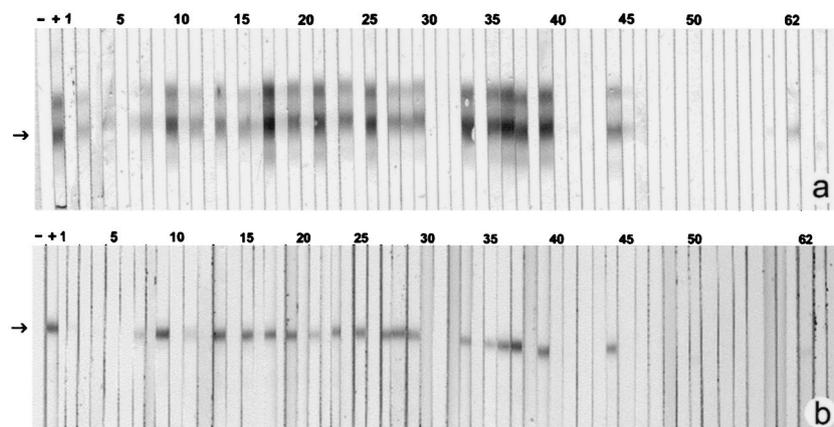


FIG. 1. Comparison of WB results for 60 human sera from France plus one negative (-) and one positive (+) control using AffEm18 (a) and RecEm18 (b). The arrow indicates Em18. All AE sera except serum 31 (from the patient with the inactive case) showed strong responses. Seven others showing weak responses were from six CE patients (samples 2, 4, 6, 16, 18, and 45) and one PE patient (sample 62) in AffEm18 WB (a), and only two (samples 2 and 62) showing a very faint response to RecEm18 (b). The numbers above the strips are sample numbers.

15). Cutoff values for AffEm18 ELISA and RecEm18 ELISA were determined as four times the optical density (OD) of a pool of serum samples from 40 healthy Japanese adults. EchWB IgG was carried out as specified by the manufacturer in France (15). Briefly, the WB banding patterns are differentiated into six groups, P1 to P5 and Neg; P1 and P2 are specific to CE; P3 is specific to AE, P4 and P5 are either CE or AE, and Neg is neither AE nor CE.

Serum samples. A total of 60 human sera, numbered from 1 to 66 (lacking numbers 55, 57, 59, 61, 63, and 65) was shipped to AMC with no clinical background information included. After performing serological examinations at AMC, the investigators were informed that the samples included 19 active AE and 1 inactive AE after treatment (see Results), 35 CE, 3 neurocysticercosis (NCC), 1 polycystic echinococcosis (PE) due to *E. vogeli*, and 1 false-positive sample found at mass screening. Surgical and pathological confirmations were carried out on all patients whose sera were tested, either in Paris or in Besançon, France.

RESULTS

AffEm18 WB and AffEm18 ELISA. Figure 1a shows the results obtained using AffEm18 WB. Although we could obtain a single band of Em18 by PIEF (9, 10, 11), AffEm18 WB showed two bands, the lower of which was Em18. As summarized in Fig. 1a and Table 1, all 19 strongest responders (3+) were patients with active AE which showed the P3 profile in EchWB IgG. However, seven weak responders were found from six patients with CE (samples 2, 4, 6, 16, 18, and 45) and one with PE (sample 62). The CE samples showed the P2 profile except for one (sample 4), which showed P5, whereas

TABLE 1. Summary of the number of seropositive samples by five different serological tests on 60 human sera^a

Disease and no. of cases	No. of seropositive samples in:				
	AffEm18 WB	AffEm18 ELISA (cutoff, 0.21)	RecEm18 WB	RecEm18 ELISA (cutoff, 0.23)	EchWB IgG (patterns 1-5) ^b
AE (20)	19 (3+) 1 ^c (-)	18 ^d (≥ 0.56) 1 ^e (0.09)	19 (3+) 1 ^c (-)	19 (≥ 0.42) 1 ^e (0.25)	19 (P3) 1 ^e (Neg) ^g
CE (35)	1 ^f (1+) 5 (1+) 29 (-)	1 ^f (0.27) 34 (<0.21)	1 ^f (1+) 34 (-)	3 (0.37, ^f 0.36, 0.25) 32 (<0.23)	16 ^f (P2) 11 (P1) 3 (P1 or P2) 5 (P5)
PE (1)	1 (1+)	1 (0.22)	1 (1+)	1 (0.28)	1 (P3)
NCC (3)	3 (-)	3 (<0.21)	3 (-)	3 (<0.23)	1 (P5) 2 (Neg)
FP ^c (1)	1 (-)	1 (0.06)	1 (-)	1 (0.08)	1 (Neg)

^a AffEm18 WB and RecEm18 WB [scores with negative (-) to strong positive (3+)] (Fig. 1), AffEm18 ELISA and RecEm18 ELISA (cutoff OD₄₀₅, 0.21 and 0.23, respectively [Fig. 2]) were carried out in Japan, and EchWB IgG (patterns 1 to 5) was carried out in France.

^b See reference 15.

^c False positive during mass screening.

^d Serum sample from patient 33 was not tested in AffEm18 ELISA due to the lack of a sufficient volume of sample.

^e Inactive AE after treatment (patient 31); EchWB IgG was positive (P3) at diagnosis before treatment.

^f With numerous cysts in the liver (patient 2).

^g Negative.

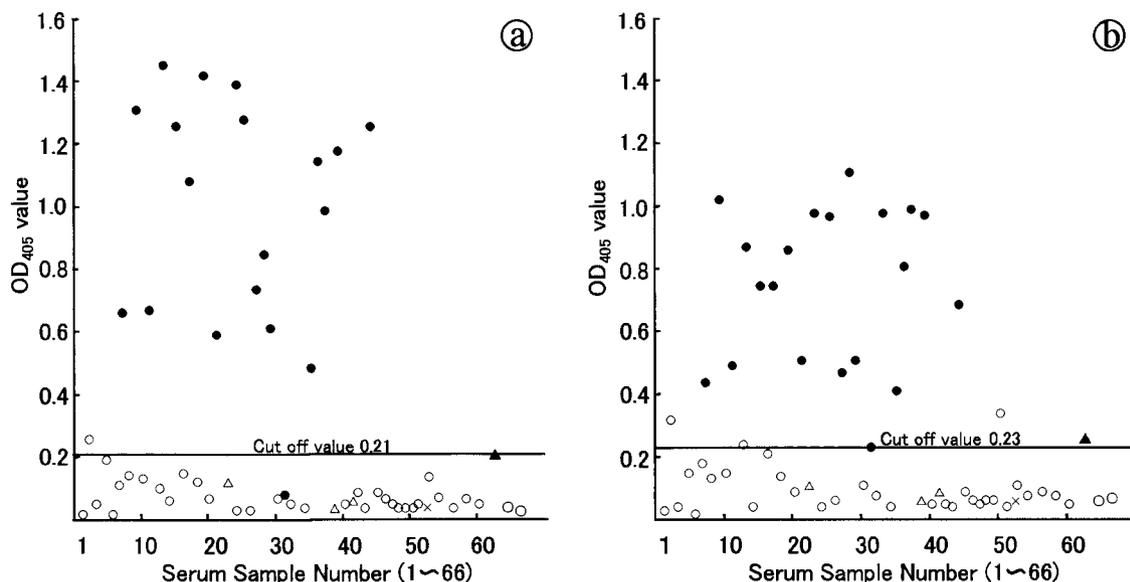


FIG. 2. Comparison of ELISA results using AffEm18 (a) and RecEm18 (b) with 60 human sera from France in a blind test. Solid and open circles indicate 20 AE and 35 CE cases, respectively. Solid and open triangles indicate 1 PE and 3 NCC cases, respectively. \times indicates one false-positive result. Sample 2 is from a CE patient with numerous cysts in the liver, and sample 31 is from a patient with inactive AE after treatment. Serum sample 33 in panel b (AE strongly positive [Fig. 1]) was not tested in AffEm18 ELISA (a) due to the lack of a sufficient volume of sample.

one PE sample (sample 62) showed a P3 profile similar to that of all the AE samples in EchWB IgG. Sample 2 was from a Turkish CE patient with numerous cysts in the liver. Sample 4 was from a patient with CE in the liver from whom the CE lesion was partially removed in Algeria in 1979 but for whom a clinical recurrence occurred in France in 1990 (the number of cysts removed in Algeria was unknown); at the time of sampling (1999), he had one hepatic CE with biliary involvement, and protoscoleces were found in the cyst fluid. Sample 6 was from a patient with two cysts in the liver. Sample 16 was from a patient with bone and muscle CE. Sample 18 was from a patient with one small ovarian cyst with anaphylaxis following surgery. Sample 45 was from the same patient (patient 18) without recurrence 3 years after stopping albendazole therapy. Figure 2a shows the results obtained using AffEm18 ELISA. Using this test, it was much easier to differentiate the 19 patients with active AE from others including the one with inactive AE (patient 31). With all 19 active-AE samples, an OD_{405} higher than 0.56 was observed, whereas with one CE sample (sample 2; OD_{405} , 0.27) and one PE sample (sample 62; OD_{405} , 0.22) the OD_{405} was at the borderline of the cutoff value (0.21). The inactive-AE sample posttreatment (sample 31) was negative.

RecEm18 WB and RecEm18 ELISA. Figure 1b shows the results obtained using RecEm18-WB. Samples from all 19 AE patients showed a strong clear band (3+), and samples from 1 CE patient (sample 2) and 1 PE patient (sample 62) showed a very faint band (1+) similar to that observed using AffEm18 WB. Figure 2b shows the results obtained using RecEm18 ELISA. The patterns from AffEm18 ELISA (Fig. 2a) and from RecEm18 ELISA (Fig. 2b) appeared to be very similar except for the one inactive-AE patient (sample 31; OD_{405} , 0.25) and two additional CE patients (samples 11 and 50; OD_{405} , 0.25

and 0.36, respectively), which became weakly positive. All other sera from patients with active AE were higher than 0.42 under the cutoff value at 0.23. Patient 31 had a progressive AE (average size of the lesions, 7/4 cm, confirmed histologically) diagnosed in 1999; the curative nature of the partial hepatectomy performed in 1999 was, however, doubtful. From 1999 to 2000, the patient was treated with albendazole, and from the beginning of 2000, the treatment was switched to mebendazole. So far, there has been no recurrence, and the antibody level, quite significant with a P3 profile using EchWB IgG at diagnosis, became very low and then negative using the same test after treatment.

DISCUSSION

Em18 WB, using either AffEm18 or RecEm18, appears to be as reliable as EchWB IgG. The overall results can be summarized as follows. (i) EchWB IgG identified 19 active AE cases correctly and 1 PE case as AE with the banding pattern P3, 30 CE cases as CE (P1 or P2), and one NCC and 5 CE cases as either AE or CE (P5); (ii) AffEm18 WB detected 19 AE cases showing very strong responses and 6 CE and 1 PE cases as AE showing weak responses; (iii) RecEm18 WB detected 19 AE cases as strong positive and 1 CE case (from sample 2) and 1 PE case (from sample 62) as very weak positive, similar to AffEm18-WB; and (iv) AffEm18 ELISA and RecEm18 ELISA differentiated 18 AE cases (lack of data from sample 33 [strong positive AE, due to lack of sufficient volume sample]) and 20 AE cases from all others, respectively, and the specificity and sensitivity of the former appeared to be very similar to but a little bit higher than those of the latter. The AE case (from sample 31) which was thoroughly negative at AMC except in RecEm18 ELISA (which gave a borderline result) could be

considered an inactive AE case following treatment (2, 12). In abortive cases, Em2 ELISA remains positive but becomes negative when Em II/3-10 ELISA or Em18 WB is used (3, 12). After surgery or long-term medical treatment, very specific antibodies have also been shown to disappear earlier while those detected using cross-reacting *Echinococcus* antigens remained longer (1).

Based on these results, it may be concluded that (i) AffEm18 WB, RecEm18 WB, and EchWB IgG are highly reliable for detection of AE, although they are not completely species specific (13); (ii) EchWB IgG is useful for detection of both AE and CE in Europe; and (iii) none of the three tests can qualitatively differentiate PE patients from AE patients. However, we expect that AffEm18 ELISA or RecEm18 WB may more easily differentiate AE from other diseases, including CE, especially in epidemiological surveys. Since PE is endemic exclusively in Latin America but not in the Northern Hemisphere, serological differentiation of AE and PE is not required for epidemiological studies. However, we need more samples of PE for evaluation of antibody responses in patients with PE.

The antibody response to Em18, which has been conceived to be highly specific to AE, is now considered to be at least in part ascribed to the critical differences in antigen release or presentation of Em18 in patients with AE, CE, and PE, due to different pathological features of *Echinococcus* infections. The *E. granulosus* metacestode grows in a double-walled cyst by endogenous budding: the outer layer is formed by fibrous tissue from the host and the inner laminated layer of the parasite. In contrast, the *E. multilocularis* metacestode grows by exogenous budding and has the potential to spread to contiguous vital structures, and the parasite tissue lacks a clear barrier from the adjacent host tissues. So far, the CE cases which showed antibody responses to Em18 were those with complicated or multiple cysts exclusively, but not those with a single cyst (13). Therefore, it is rather easy to expect similar responses from PE cases involving polycystic lesions. This study also recorded that the CE patient showing the highest response (patient 2) had numerous cysts in the liver. Although these CE cases were seropositive by AffEm18 WB and RecEm18 WB, it is evident that the response was much weaker than that of AE cases and that the OD value in ELISA was also much lower than for AE cases (7, 13). EchWB IgG using a single strip should be highly reliable in Europe, although a minority of CE cases may exhibit P4 or P5 profiles, which are serologically indistinguishable from AE. Minor cross-reactivity with neurocysticercosis and schistosomiasis has also been reported; however, such patients are basically rare in Europe except among immigrants or refugees from Latin America, Africa, or Asia (5-7).

The sensitivity of Em18 WB using Em18 purified by PIEF or affinity chromatography using polyclonal antibodies against Em18 should be more reliable in detecting AE patients in Japan than are other serological assays using crude antigens for ELISA and WB. The AMC group was asked by clinicians to check sera from 33 persons suspected of having AE in Japan over the last 2 years. AMC identified 28 patients as having AE and 5 others as not having AE based on the simple criterion of the presence or absence of an antibody response to Em18 by WB. All these were later confirmed by pathological testing to

be 100% correct. The non-AE sera were from two patients with hemangioma, one with a hepatic cyst, one with fascioliasis, and one with CE who was born and resided until 5 years old in Argentina (9, 11). Em18 serology using either WB or ELISA seems sufficient for screening and identification of AE patients in Japan and is expected to be highly useful in China, where both AE and CE are highly endemic. Both Em18-WB and EgCF (cyst fluid of *E. granulosus*) ELISA have been used for differentiation and identification of AE and CE together with ultrasound examination for epidemiological surveys in China (13, 17). EchWB IgG might be much simpler for identification of AE and CE at once in China, but the occurrence of false-positive cases in patients with schistosomiasis japonica, paragonimiasis, and other trematodiasis and cestodiasis, especially cysticercosis, all of which are still more common than echinococcosis in China, should be studied.

ACKNOWLEDGMENTS

This study was supported in part by grants-in-aid from the Japan Society of Promotion of Science (12557024 and 14256001) and by the NIH (1 R01 TW01565-01) (principal investigator, P. S. Craig) to A. Ito.

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