CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 1997, p. 57–59 Vol. 4, No. 1
1071-412X/97/$04.00+0

Immunodiagnosis of Alveolar Echinococcosis by Enzyme-Linked Immunosorbent Assay Using a Partially Purified Em18/16 Enriched Fraction†

AKIRA ITO,[1]* LIANG MA,[1,2] MAKOTO ITOH,[3] SEUNG-YULL CHO,[4] YOON KONG,[5] SHIN-YONG KANG,[6] TOSHIHIRO HORII,[7] XIN-LI PANG,[7] MUNEHIRO OKAMOTO,[8] TERUO YAMASHITA,[9] MARSHALL W. LIGHTOWLERS,[10] XIAO-GEN WANG,[2] AND YUE-HAN LIU[2]

Institute of Infectious and Parasitic Diseases, First Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing, Sichuan, People's Republic of China[2]; Department of Parasitology, Catholic University Medical College,[4] Biomedical Center, Korea Institute of Science and Technology,[5] and Department of Parasitology, College of Medicine, Chung-Ang University,[6] Seoul, Korea; Department of Parasitology, Gifu University School of Medicine, Gifu,[1] Department of Molecular Protozoology, Institute of Microbial Diseases, Osaka University,[7] and Institute of Experimental Animal Sciences, Osaka University School of Medicine,[8] Suita, and Department of Medical Biology, Medical School, Nagoya City University,[3] and Department of Virology, Aichi Prefectural Institute of Public Health,[9] Nagoya, Japan[9]; and Molecular Parasitology, Veterinary Clinical Centre, University of Melbourne, Melbourne, Victoria 3030, Australia[10]

Received 25 March 1996/Returned for modification 27 September 1996/Accepted 31 October 1996

An improved enzyme-linked immunosorbent assay (ELISA) system using partially purified Em18/16 enriched fraction (PP-Em18/16) prepared by isoelectric focusing was evaluated for serodiagnosis of alveolar echinococcosis (AE). The PP-Em18/16-ELISA was compared with Em2^{plus}-ELISA by using sera from AE and cystic echinococcosis (CE) patients in China, where both AE and CE are endemic; sera from CE patients in Australia, where only CE exists; and sera from patients with cysticercosis, paragonimiasis, or sparganosis in Korea, where no indigenous AE or CE exists. We used Em2^{plus}-ELISA as a standard ELISA and found 24.6% (17 of 69 specimens) cross-reactivity with sera from CE. Furthermore, some of the sera from paragonimiasis, sparganosis, and cysticercosis patients were also cross-reactive in the Em2^{plus}-ELISA. When we tested for similar cross-reactivity in the same sera from CE patients by PP-Em18/16-ELISA (23.2%, 16 of 69), it became evident that the specificity of the PP-Em18/16-ELISA was better than that of the Em2^{plus}-ELISA, since no sera from patients with the examined parasitic diseases except CE showed cross-reactivity. Some CE patients from China showed exceptionally high levels of antibody in comparison with those of CE patients from Australia, where no AE occurs. It is speculated that these patients with strongly positive cases of CE from China may have been exposed to both species of *Echinococcus*.

Alveolar echinococcosis (AE), caused by the larval stage of the fox tapeworm, *Echinococcus multilocularis*, is globally a rare helminth zoonosis but with significant endemicity in parts of China, Japan, Russia, central Asia, and North America. A focus also occurs in central western Europe (France, Germany, Switzerland, and Austria) (1, 2, 17). The disease has a chronic course of increasing hepatic pathology like hepatic cancer. Radical liver resection is currently the only reliable curative approach (6, 14). Therefore, immunodiagnosis for early detection of AE has been a critical requirement for reduction of mortality (14). Immunodiagnostic tests based on antibody detection exhibit high sensitivity and reasonable specificity and are useful for confirming a clinical diagnosis and for seroepidemiological studies (1–3, 6). The Em2 enzyme-linked immunosorbent assay (Em2-ELISA) using partially purified native antigen Em2 has been recommended by the World Health Organization for serodiagnosis of AE (6, 8). The same group has developed the Em2^{plus}-ELISA system, now commercially available. It consists of a cocktail of the native Em2 antigen and a recombinant *E. multilocularis* molecule (II/3-10) that was expected to be highly specific to this parasite (5, 18). It was reported to have high sensitivity and specificity (>95%) (7). The instructions attached to the commercially available Em2^{plus}-ELISA kit, however, indicate that the test is not completely specific for AE and cross-reacts with approximately 25% of sera from patients infected with cystic echinococcosis (CE), caused by the larval stage of the dog tapeworm, *Echinococcus granulosus*, since recent study has revealed that the protein molecule II/3-10 is known to be shared by *E. multilocularis* and *E. granulosus* (4).

Recently, Ito and colleagues reported that an antibody response against a previously undescribed protein epitope, designated Em18, which was easily detectable by Western blotting (immunoblotting), was a good serologic marker either for differentiation of AE from other parasitoses, including CE, or for differentiation of active from inactive cases of AE (9, 11, 12). Other studies have also confirmed that Em18 is the most species-specific epitope as determined by Western blotting for immunodiagnosis of human AE (19, 20). Based on these observations, we have tried to establish an improved ELISA system using partially purified Em18/16 enriched fraction (PP-Em18/16) in comparison with the commercially available standard Em2^{plus}-ELISA system.

* Corresponding author. Mailing address: Department of Parasitology, Gifu University School of Medicine, Gifu 500, Japan. Phone: 81-58-265-1241, ext. 2252. Fax: 81-58-267-2960. E-mail: akiraito@cc.gifu-u.ac.jp.

† This paper is dedicated to Keiko Mori, Gifu University School of Medicine, who died by a sudden heart attack on 18 July 1995, lest we forget her great help and smile.

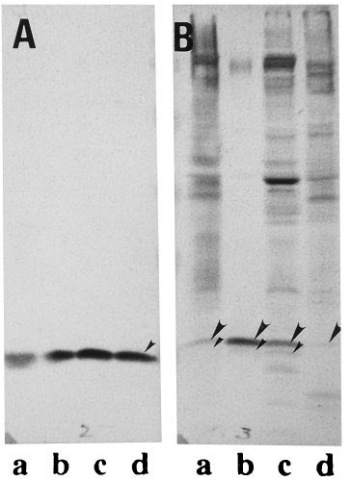

FIG. 1. Western blots of PP-Em18/16 compared with crude antigens. Lanes a, c, and d, different batches of crude antigens; lanes b, fraction PP-Em18/16. (A) Result of treatment with monoclonal antibody against Em16. (B) Result of treatment with AE patients' pooled serum. Em18 and Em16 are indicated (large and small arrowheads, respectively).

MATERIALS AND METHODS

ELISA and Western blotting. Em2^{plus}-ELISA kits (no. 9300) were purchased from Bordier Affinity Products, Crissier, Switzerland, whereas PP-Em18/16 was obtained from protoscoleces of *E. multilocularis* by isoelectric focusing. Protoscoleces of *E. multilocularis* (2.0 ml of packed protoscoleces [approximately 2×10^5]) were washed in 0.15 M phosphate-buffered saline (PBS) (pH 7.4) several times and kept at −30°C. Frozen protoscoleces, resuspended in 10 ml of PBS with 2.5 mg of aprotinin (Sigma) per ml, were homogenized in a glass-Teflon pestle homogenizer on ice and sonicated on ice (10 s [10 μm, peak to peak] on and 5 s off) for 5 min. The sonicate was left standing on ice for 2 h prior to centrifugation at 12,000 × *g* for 30 min at 4°C. The crude antigen, dialyzed against 5 mM Tris-HCl buffer (pH 7.2) to remove excess salts, was subjected to isoelectric focusing. PP-Em18/16 was prepared as several pooled fractions of pH 5.2 to 5.5 after the presence of both Em18 and Em16 was checked by Western blotting (9, 11, 12) The fractions contained Em18 and Em16 as the two major components (Fig. 1). Maxisorb (Nunc, Roskilde, Denmark) was used for immunoplates similar to those used for Em2^{plus}-ELISA. Ten micrograms of PP-Em18/16 antigen was used to coat each well as previously described (7). All serum samples were diluted 1/200 with PBS containing 0.05% Tween 20 (0.1 M; pH 7.4). The specific immunoglobulin G antibody in serum was detected with anti-human immunoglobulin G-alkaline phosphatase and -peroxidase conjugates; the substrates were *p*-nitrophenyl-phosphate and 4-chloro-1-naphthol, respectively. The absorbances of Em2^{plus}-ELISA and PP-Em18/16-ELISA were read at 405 nm and 492 nm, respectively. Em2^{plus}-ELISA was carried out according to the manual provided by Bordier Affinity Products (7). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were carried out in order to show the most species-specific band, Em18, by using commercially available precast 18% isocratic and 4 to 20% gradient gels (no. 01-102, -106, -022, and -026, SDS-PAGE Mini; TEFCO, Tokyo, Japan) and crude antigen and/or PP-Em18/16 as previously described (9, 11, 12) (Fig. 1). Serum samples were diluted 1/50.

Serum samples. We used a total of 127 unknown serum samples from China (according to the background information obtained after the blind ELISA carried out in Japan, they consisted of 79 follow-up study samples from 24 AE patients and 48 follow-up study samples from 34 CE patients from Sichuan Province or Xingjiang Autonomous Region, China, where AE and CE are highly endemic); 21 CE patient sera from Australia, where only CE is endemic, with high titers in ELISA (maximum, 1:1,600) and immunoelectrophoresis (seven bands including arc 5, the maximum) by standard serologic methods for CE in Australia (16); and 28 cysticercosis, 2 sparganosis, and 5 paragonimiasis samples from Korea (where no indigenous CE or AE exists). All serum samples examined were from patients whose infections had been confirmed surgically, for echinococcosis patients, or parasitologically, for patients from China, Korea, or Australia. Sera were shipped to Gifu, Japan, either lyophilized or frozen. One normal serum pool from 10 Japanese volunteers and one commercially available control for the Em2^{plus}-ELISA kit were used as controls.

TABLE 1. Sensitivity and specificity of PP-Em18/16- and Em2^{plus}-ELISAs for differentiation of AE from CE and other parasitic diseases

Patient type (*n*)	Em2^{plus}-ELISA			PP-Em18/16-ELISA		
	No. of positive samples	No. of negative samples	%[a]	No. of positive samples	No. of negative samples	%[a]
Chinese AE (24)[b]	21	3	87.5	24	0	100
Chinese AE (79)[c]	63	16	79.7	72	7	91.1
Chinese CE (34)[b]	11	23	32.4	12	22	35.3
Chinese CE (48)[c]	15	33	31.3	16	32	33.3
Australian CE (21)	2	19	9.5	0	21	0.0
Cysticercosis (28)[d]	1	27	3.6	0	28	0.0
Sparganosis (2)[d]	0	2	0.0	0	2	0.0
Paragonimiasis (5)[d]	3	2	60.0	0	5	0.0

[a] Percent positive.
[b] Sera from 24 AE and 34 CE patients were obtained prior to chemotherapy with albendazole.
[c] The 79 AE and 48 CE samples were follow-up study samples from the 24 AE and 34 CE patients, respectively.
[d] From Korea.

RESULTS

Western blots of PP-Em18/16 and crude antigens are shown in Fig. 1. PP-Em18/16 showed only two major bands. The major and relatively minor bands detected by AE patients' pooled serum were against Em18 and Em16, respectively. The major band detected by a monoclonal antibody was against Em16. We examined 79 Chinese AE samples (from 24 patients), 48 Chinese CE samples (from 34 patients), 21 Australian CE samples, and 35 other parasitosis samples from Korea. The results are summarized in Table 1. When a cutoff value (absorbance at 0.6) for the Em2^{plus}-ELISA was determined with the weakly positive serum supplied with the kit, there were false-positive reactions for CE (17 of 69 samples; 24.6%), paragonimiasis (3 of 5 samples; 60%), sparganosis (1 of 2 samples, 50%), and cysticercosis (1 of 28 samples; 3.6%). Four CE samples from three Chinese patients (4 of 17) showed exceptionally high values in both Em2^{plus}- and PP-Em18/16-ELISAs, whereas all other CE samples (13 of 17) showed values relatively lower than those for AE. As the Em2^{plus}-ELISA was the only ELISA commercially available, we tried to evaluate our own improved method compared with its false positiveness. In order to test for similar false positiveness for CE, the cutoff value in the PP-Em18/16-ELISA was set at an absorbance of 1.0. The false-positive reactions disappeared except for CE patient samples from China that remained unchanged (16 of 69; 23.2%); false-positive cases of CE from Australia and of paragonimiasis, sparganosis, and cysticercosis tested negative (Table 1).

DISCUSSION

These results revealed that the reliability of serodiagnosis of AE by PP-Em18/16-ELISA was better than that of Em2^{plus}-ELISA in its specificity (Table 1). The comparison of false-positive reactions in the Em2^{plus}-ELISA and the PP-Em18/16-ELISA with sera from patients with other parasitoses, including paragonimiasis, cysticercosis, and sparganosis, revealed a critical difference between these two serological methods (Table 1). Although the cutoff for the PP-Em18/16-ELISA (absorbance at 1.0) was determined on the basis of similar false positiveness for CE by Em2^{plus}-ELISA, all AE patients before treatment with albendazole for follow-up study were found positive by the PP-Em18/16-ELISA (100% of 24 AE patients),

and this ELISA was better than the Em2^{plus}-ELISA in its sensitivity (87.5% [21 of 24 AE patients). All 79 AE sera, including 7 which were negative by the cutoff at 1.0 in the PP-Em18/16-ELISA, were still antibody positive against Em18 as determined by Western blotting (15). As these seven negative AE sera were expected to be from patients who were cured or at least greatly improved after long-term treatment with albendazole (14a), the PP-Em18/16-ELISA may be more useful than Western blotting for monitoring of prognosis. As Em16 is known to be shared by AE and CE (12), the cutoff value in the PP-Em18/16-ELISA was made higher than that in the Em2^{plus}-ELISA. At this stage, at which we cannot purify Em18 without Em16, the low cutoff value in the Em2^{plus}-ELISA has higher sensitivity and more reliability for monitoring of AE patients provided that AE has been confirmed by detection of an antibody response against Em18 by Western blotting, etc. (10). When we drew the lower cutoff value at 0.8, the PP-Em18/16-ELISA still had better resolution for differentiation of AE and CE from other parasitoses. All sera were still negative for paragonimiasis, sparganosis, or cysticercosis, but 10 more CE sera (25 of 48 versus 15 of 48) and 5 more AE sera (77 of 79 versus 72 of 79) became positive.

False-positive rates for CE patient sera from China by both the Em2^{plus}-ELISA and the PP-Em18/16-ELISA were higher (three times) than those for sera from Australia, where no AE exists. The four CE samples which showed exceptionally high absorbances by Em2^{plus}-ELISA as well as by PP-Em18/16-ELISA were especially interesting, since all CE samples from Australia showed much lower absorbances and, according to the manual for Em2^{plus}-ELISA, CE-positive cases used to show even lower values. These four CE sera from three patients and six other CE sera from six patients in China were exceptionally antibody positive against Em18 as determined by Western blot analysis, similar to all AE sera examined (15). There are reports of some CE patient sera from Xingjiang Autonomous Region, where both AE and CE are highly endemic (1), and a case of double infection of AE and CE (21) showing an antibody response against Em18 similar to an AE response (13), whereas no CE sera from Libya and Uruguay, where only CE occurs, reacted with Em18 (20). All the Chinese patients examined in the present study came from Xingjiang Autonomous Region or Sichuan Province, where AE and CE are highly endemic. Therefore, we speculate that these 10 samples from nine CE cases in China might be from patients who have been exposed to *E. multilocularis* as well as *E. granulosus* (20). It may be possible, but the percentage of suspected doubly exposed cases is too high (26.5% [9 of 34]). Alternatively, we may speculate that these CE cases, at least other than the three cases (four samples) showing exceptionally high values, had some unique false positiveness due to the side effect of pretreatment with albendazole in cyst breakdown and exposure of cross-reactive antigens. If the latter is the case, Em18 should also be shared between all AE patients and a minority of CE patients. Further evaluation, especially from clinical study including imaging and comparative study of antigenicity of protoscoleces of both species, is required to confirm these speculations.

The PP-Em18/16-ELISA reported here has advantages over the commercially available Em2^{plus}-ELISA in its specificity for differentiation of AE from other parasitoses, including CE, paragonimiasis, and cysticercosis, although the sensitivity (cutoff value) appeared to be somewhat lower in the former than in the latter. The specificity and sensitivity in serodiagnosis for AE should be improved when we can purify Em18 by affinity purification using a monoclonal antibody against Em18 or when we can produce Em18 as a recombinant antigen. It is anticipated that an Em18-ELISA would be highly specific to AE.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ohyama Health Foundation, the Uehara Memorial Foundation, and the Nissan Science Foundation; by grants-in-aid (06044089 and 07044243) for Scientific Research (International Scientific Research Program, Joint Research) from the Ministry of Education, Science and Culture, Japan, to A. Ito; and by a grant from the International Collaboration Project (1995), Ministry of Science and Technology, Korean Government, to Y. Kong.

REFERENCES

1. **Craig, P. S., L. Deshan, C. N. L. Macpherson, S. Dazhong, D. Reynolds, G. Barnish, B. Gottstein, and W. Zhirong.** 1992. A large focus of alveolar echinococcosis in central China. Lancet **340:**826–831.
2. **Craig, P. S., L. Deshan, and D. Zhaoxun.** 1991. Hydatid disease in China. Parasitol. Today **7:**46–50.
3. **Craig, P. S., M. T. Rogan, and J. C. Allan.** 1996. Detection, screening and community epidemiology of taeniid cestode zoonoses: cystic echinococcosis, alveolar echinococcosis and neurocysticercosis. Adv. Parasitol. **38:**170–250.
4. **Felleisen, R., and B. Gottstein.** 1994. Comparative analysis of full-length antigen II/3 from *Echinococcus multilocularis* and *E. granulosus*. Parasitology **109:**223–232.
5. **Frosch, P. M., M. Frosch, T. Pfister, and V. Schaad.** 1991. Cloning and characterization of an immunodominant major surface antigen of *Echinococcus multilocularis*. Mol. Biochem. Parasitol. **48:**121–130.
6. **Gottstein, B.** 1992. *Echinococcus multilocularis* infection: immunology and immunodiagnosis. Adv. Parasitol. **31:**321–380.
7. **Gottstein, B., P. Jacquier, S. Bresson-Hadni, and J. Eckert.** 1993. Improved primary immunodiagnosis of alveolar echinococcosis in humans by an enzyme-linked immunosorbent assay using the Em2^{plus} antigen. J. Clin. Microbiol. **31:**373–376.
8. **Gottstein, B., P. M. Schantz, and J. F. Wilson.** 1985. Serological screening for *Echinococcus multilocularis* infections with ELISA. Lancet **i:**1097–1098.
9. **Ito, A., M. Nakao, H. Kutsumi, M. W. Lightowlers, M. Itoh, and S. Sato.** 1993. Serodiagnosis of alveolar hydatid disease by Western blotting. Trans. R. Soc. Trop. Med. Hyg. **87:**170–172.
10. **Ito, A., Y. Osawa, M. Nakao, T. Horii, M. Okamoto, M. Itoh, and T. Yamashita.** 1995. Em18 and Em16, new serologic marker epitopes for alveolar echinococcosis in Western blot analysis, are the only two epitopes recognized by commercially available weak positive (cut off) sera for Em2^{plus}-ELISA. J. Helminthol. **69:**369–371.
11. **Ito, A., P. M. Schantz, and J. F. Wilson.** 1995. Em18, a new serodiagnostic marker for differentiation of active and inactive cases in alveolar hydatid disease. Am. J. Trop. Med. Hyg. **52:**41–44.
12. **Ito, A., X. Wang, and Y. Liu.** 1993. Differential serodiagnosis of alveolar and cystic hydatid disease in the People's Republic of China. Am. J. Trop. Med. Hyg. **49:**208–213.
13. **Ito, A., H. Wen, P. S. Craig, M. Nakao, T. Horii, M. Okamoto, M. Itoh, Y. Osawa, X. Wang, and Y. Liu.** Antibody responses against Em18 and Em16 serodiagnostic markers for alveolar and cystic echinococcosis patients from northwest China. Southeast Asian J. Trop. Med. Pub. Health, in press.
14. **Kasai, Y., I. Koshino, N. Kawanishi, H. Sakamoto, E. Sasaki, and M. Kumagai.** 1980. Alveolar echinococcosis of the liver. Ann. Surg. **191:**145–152.

14a. **Ma, L., et al.** Unpublished data.

15. **Ma, L., A. Ito, Y. Liu, X. Wang, Y. Yao, and D. Yu.** Evaluation of the diagnostic value of 18/16 kDa antigens in *Echinococcus multilocularis* by Western blotting. Chinese J. Parasitol. Parasit. Dis., in press.
16. **Rickard, M. D., R. D. Honey, J. L. Brumley, and G. F. Mitchell.** 1984. Serological diagnosis and post-operative surveillance of human hydatid disease. II. The enzyme-linked immunosorbent assay (ELISA). Pathology **16:** 211–215.
17. **Schantz, P. M., B. Gottstein, R. Amman, and A. Lanier.** 1991. Hydatid and the Arctic. Parasitol. Today **7:**576–585.
18. **Vogel, M., B. Gottstein, N. Muller, and T. Seebeck.** 1988. Production of a recombinant antigen of *Echinococcus multilocularis* with high immunodiagnostic sensitivity and specificity. Mol. Biochem. Parasitol. **31:**117–125.
19. **Wen, H., and P. S. Craig.** 1994. IgG subclass responses in human cystic and alveolar echinococcosis. Am. J. Trop. Med. Hyg. **51:**741–748.
20. **Wen, H., P. S. Craig, A. Ito, D. A. Vuitton, S. Bresson-Hadni, J. C. Allan, M. T. Rogan, E. Paollilo, and M. Shambesh.** 1995. Immunoblot evaluation of IgG and IgG subclass antibody responses for immunodiagnosis of human alveolar echinococcosis. Trop. Med. Parasitol. **89:**485–495.
21. **Wen, H., W. L. Tian, P. F. Zou, and M. X. Xiang.** 1992. A rare case of mixed cystic and alveolar hydatidosis. Trans. R. Soc. Trop. Med. Hyg. **86:**290–291.