

Development of an Immunochromatographic Test To Detect Antibodies against Recombinant Em18 for Diagnosis of Alveolar Echinococcosis[∇]

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Received 1 August 2008/Returned for modification 17 September 2008/Accepted 29 October 2008

An immunochromatographic test (ICT) for the rapid detection of antibodies to *Echinococcus multilocularis* was developed. The ICT showed a sensitivity of 94% and a specificity of 95.4%. High degrees of agreement were observed between the ICT and an enzyme-linked immunosorbent assay ($\kappa = 0.93$) and between the ICT and immunoblot analysis ($\kappa = 0.97$). It is expected that the ICT developed in this study will be useful for the serodiagnosis of alveolar echinococcosis.

Alveolar echinococcosis (AE), caused by the larval stage of *Echinococcus multilocularis*, is a serious parasitic disease of humans in countries of the higher latitudes of Northern Hemisphere. In the previous decade, a lot of new data have been published on prevalence of *E. multilocularis* in final and intermediate hosts in areas where it had previously not been recorded (5). Humans are accidentally infected with *E. multilocularis* by ingestion of eggs excreted with the feces of carnivores harboring adult tapeworm of this species. It is thought that humans become exposed to *E. multilocularis* by handling of infected definitive hosts or by ingestion of food contaminated with eggs. Oncospheres hatched from eggs in the small intestine of humans migrate via the portal system into various organs, mainly the liver, and differentiate and develop into the metacestode stage. The metacestodes propagate asexually like a tumor, leading to organ dysfunction. Since clinical symptoms usually do not become evident until 10 or more years after initial parasite infection, early diagnosis and treatment especially during asymptomatic period are important for reduction of morbidity and mortality (14). About a third of patients have cholestatic jaundice, and about a third of patients have epigastric pain. In the remaining patients, *E. multilocularis* infections are incidentally detected during medical examination for symptoms such as fatigue, weight loss, and hepatomegaly (15). At present, diagnosis of AE is primarily based on imaging techniques including echography, computed tomography, magnetic resonance imaging, and positron emission tomography with [¹⁸F]fluoro-deoxyglucose (3). However, these imaging techniques are sometime limited by the small size of visualized lesions and atypical images, which are difficult to distinguish from abscesses or neoplasms. Moreover, these imaging techniques are unsuitable for diagnosis in isolated communities. Therefore, immunological tests have been considered important methods to confirm clinical findings, to give diagnostic help by providing information on the parasite in case of unclear images, or to survey in areas of endemicity

where imaging techniques are not readily available (4, 9, 11). Previously, we have reported an enzyme-linked immunosorbent assay (ELISA) and an immunoblot analysis (IB) by using recombinant *E. multilocularis* 18-kDa antigen (Em18), the breakdown product of ezrin-radixin-moesin-like protein (2) that is also known as EM10 (8), EM II/3 (7), or EM4 (10) by the cysteine peptidase, and demonstrated that these two tests have a high potential for differentially diagnosing AE (1, 12, 16, 18). However, these two methods are time-consuming and require special materials and equipments, which make them not suitable for clinical applications. In contrast, an immunochromatographic test (ICT) is a simple, rapid, and reliable method for detection of specific antibodies to infectious agents. In the present study, we developed an ICT with rEm18 antigen for diagnosis of AE and compared ICT with ELISA and IB.

The rEm18 was expressed in a bacteria system as described previously (16) with some modifications. Briefly, a DNA fragment encoding the Em18 was amplified by PCR with the primers 5'-GGGAATTCAAGGAGTCTGACTTAGCGGAT-3' and 5'-TTGGATCCTAGGGCTTCACTTTCATCATCC TG-3'. The PCR products were digested with EcoRI and BamHI and cloned into bacterial expression vector pTWIN-1 (New England Biolabs, Beverly, MA) for producing a fusion protein with chitin binding domain/mini-inteins. The cloned plasmid was transfected into *Escherichia coli* ER2566 strain and expression of the recombinant protein was induced by the addition of 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) to the culture. The expressed rEm18 was purified by using a chitin column (New England Biolabs) according to the manufacturer's instructions. The purified rEm18 did not have the fusion partner, because rEm18 was released by intein activity of the fusion partner itself during purifications (6). The purified rEm18 (1 mg/ml) and anti-goat immunoglobulin G (IgG) antibody (1 mg/ml) were sprayed onto a nitrocellulose membrane in a 1-mm-wide line as test and control lines, respectively. The nitrocellulose membrane with rEm18 and anti-goat IgG antibody, absorbent pad, and substrate reservoir pad were assembled on a laminated membrane card, and the assembled sheet was cut into strips 5 mm in width. The strip was placed into a plastic assay device (Mitsubishi Chemical Medience, Tokyo, Japan) with windows for applying a serum sample and

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[∇] Published ahead of print on 5 November 2008.

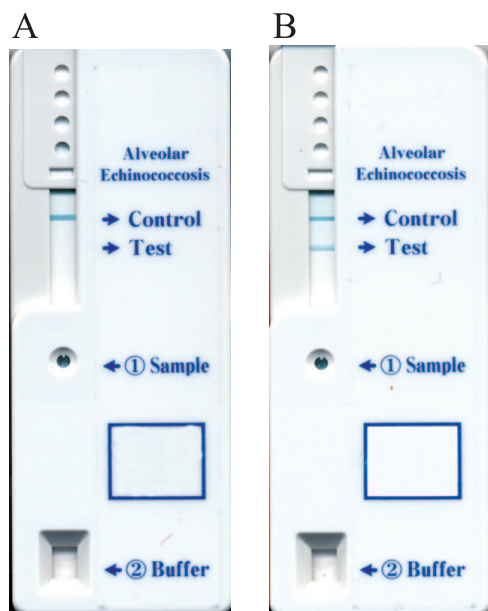


FIG. 1. Examples of ICT tests with negative and positive sera. (A) Result with a negative serum (one blue band at control line in the result window); (B) result with a positive serum (two blue bands at control and test lines in the result window). The inscriptions “Sample” and “Buffer” represents the positions for loading of sample and of substrate solution, respectively.

a substrate solution. For assay, first, 10 μ l of serum sample was mixed 20 μ l of a serum dilution buffer containing 0.1 mg of alkaline phosphatase-conjugated anti-human IgG antibody (Dako, Tokyo, Japan)/ml in a tube, and the mixed serum sample was applied into the sample window of the plastic device. Soon after application of the serum sample (within 30 s), 200 μ l of the substrate solution was loaded onto the substrate reservoir pad, and the result was evaluated after 20 min. BCIP (5-bromo-4-chloro-3-indolylphosphate) was used for color development. As shown in Fig. 1, a sample was considered positive if two color lines corresponding to rEm18 and anti-goat IgG antibody appeared in the result window, and a sample was considered as negative if one color line corresponding to anti-goat IgG antibody appeared in the result window. In cases where there was no appearance of a colored anti-goat IgG antibody line, the assay was invalid even if a colored rEm18 line appeared. ELISA and IB were performed as described previously (16), except using the rEm18 prepared in the present study.

A total of 94 serum samples, including 50 serum samples from AE patients, 24 serum samples from cystic echinococcosis (CE) patients, and 20 serum samples from healthy persons, were examined by ICT, ELISA, and IB. Each diagnosis of AE and CE had been carried out by imaging techniques, clinical findings, histological observations (if feasible), and/or serology of IB with recombinant Em18 (16) or EmAgB8/1 (13). As shown in Table 1, 47 AE and 2 CE patient sera were determined to be positive by ICT, and none of sera from healthy persons showed positive reactions; thus, the sensitivity and specificity of ICT were 94.0 and 95.4%, respectively. There were no significant differences in sensitivity and specificity among ICT, ELISA, and IB ($P > 0.1$, Pearson chi-square test).

TABLE 1. Results of ICT, ELISA, and IB with sera from AE patients, CE patients, and healthy persons

Serum sample source	Total no. of samples examined	Samples examined by:					
		ICT		ELISA		IB	
		No. positive	%	No. positive	%	No. positive	%
AE patient	50	47	94.0	47	94.0	47	94.0
CE patient	24	2	8.3	1	4.2	3	12.5
Healthy subject	20	0	0	0	0	0	0

Two CE patient sera, determined to be positive by ELISA and/or IB, were also positive by ICT. This is not an incomprehensible result, because it is known that a few CE patient sera react to rEm18 even though rEm18 is highly specific antigen for AE (11, 12, 16, 18). These results indicated that the ICT is a sensitive and specific method for the diagnosis of *E. multilocularis* infection.

The results obtained by ICT were compared to those of previously established ELISA and IB with rEm18 (Table 2). All ELISA-positive samples, except one from AE patient, were ICT positive. Two ELISA-negative samples with the optical density values 0.068 and 0.079 close to the cutoff optical density value of 0.093 at 405 nm were ICT positive, and both were also positive by IB (data not shown). All IB-positive samples, except for one from a CE patient, were ICT positive, and none of the IB-negative samples was ICT positive. The degrees of agreement between ICT and ELISA and between ICT and IB were estimated by kappa analysis (17). A kappa statistics value of >0.75 , 0.40 to 0.75, or <0.4 represents excellent agreement, good to fair agreement, and poor agreement, respectively. High degrees of agreement were observed between ICT and ELISA ($\kappa = 0.93$) and between ICT and IB ($\kappa = 0.97$), which indicated that ICT is reliable.

In conclusion, we developed a rapid, simple, sensitive, and specific ICT with rEm18 for detection of specific antibodies to *E. multilocularis* infection. Although ICT, ELISA and IB with rEm18 show similarities to each other with regard to both sensitivity and specificity, ICT has the following advantages: (i) expertise, experience, and special equipment are not required; (ii) 20-min incubation is enough to detect specific antibodies; and (iii) it is more economical than ELISA and IB. These advantages suggest a high diagnostic potential for the ICT in clinical practice in providing immediate and proper treatments and in mass-screening programs in areas of endemicity as a

TABLE 2. Comparison of ICT with ELISA and IB^a

Samples examined by ICT	Samples examined by:					
	ELISA			IB		
	No. positive	No. negative	Total	No. positive	No. negative	Total
Positive	47	2	49	49	0	49
Negative	1	44	45	1	44	45
Total	48	46	94	50	44	94

^a Results with a total 94 sera shown in Table 1 were used for comparisons.

primary screening tool. Further analysis on stability of ICT and a large-scale evaluation might be necessary.

This study was financially supported in part by Hokkaido Translational Research Project from the Ministry of Education of Japan on the development of the rapid test for echinococcosis (from 2007 onward) to A.I.

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