Use of Two Sensitive and Specific Immunoblot Markers, Em70 and Em90, for Diagnosis of Alveolar Echinococcosis

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Antibodies against Echinococcus multilocularis metacestodes were screened by immunoblotting sera from patients with alveolar echinococcosis (n = 39), cystic echinococcosis (n = 109), or other parasitic infections (n = 66) and healthy individuals (n = 32). Two antigens, approximately 70 and 90 kDa, are found to be valuable for confirmatory diagnosis, with a sensitivity and specificity of 100 and 99.51%, respectively.

Human alveolar echinococcosis (AE), caused by the metacestode of Echinococcus multilocularis, is considered the most potentially lethal parasitic zoonosis in the nontropical areas of the Northern Hemisphere (1, 2, 7, 15). Imaging techniques, serological tests, and biopsy are used for the diagnosis of AE. However, radiological patterns of AE lesions are difficult to interpret and may resemble primary hepatic neoplasm or metastasis (3). Diagnosis is possible when the imaging findings are correlated with appropriate clinical and serological findings (3, 20). Tests based on purified and recombinant antigens are reported to be useful in the differential diagnosis of patients infected with Echinococcus granulosus, the agent responsible for cystic echinococcosis (CE), and E. multilocularis (4, 5, 17, 18). Immunoblotting is used as a confirmatory test for excluding cross-reactivity in positive sera or in assessment of the screening test results (6, 12).

Two E. multilocularis metacestode antigens of 18 and 16 kDa (Em18 and Em16, respectively) were recommended for the specific diagnosis of AE by immunoblotting (6). Em18 was reported to be more specific and sensitive than Em16 (7) and is useful in both demonstrating active lesions (8) and evaluating the efficacy of chemotherapy (13). However, Nirmalan and Craig (14) reported that both Em18 and Em16 antigens cross-reacted with sera from CE patients. In addition, previous immunoblotting studies reported problems with band identification and indeterminate results (10, 12). Differentiating Em18 and Em16 was found to be difficult, and the location of Em16 has been identified by a specific monoclonal antibody (8, 9, 10). Purification of Em18 by affinity chromatography and production of a recombinant Em18 led to highly reliable, but not completely species-specific, diagnosis of echinococcosis by immunoblotting (11). These limitations indicated the need for new confirmatory tests in the diagnosis of AE.

In this study, antibodies against antigens of E. multilocularis metacestodes were screened for the presence of sensitive and specific markers for the serological diagnosis of AE by immunoblotting.

Serum samples from 39 patients with AE and 109 patients with CE were included in this study. Diagnosis of AE is confirmed by histopathology (n = 27), and patients with typical liver lesion morphology were determined to have advanced inoperable AE (n = 12) by clinical and radiological imaging findings. Diagnosis of CE was confirmed by serology and typical imaging findings and/or surgery. To assess cross-reactivity, serum samples were selected from patients with other proven parasitic infections, such as fascioliasis (n = 32), Taenia saginata taeniasis (n = 7), ascariasis (n = 16), and visceral leishmaniasis (n = 11). Additionally, sera from 32 healthy individuals were included in the tests. Serum samples were kept frozen at −30°C until tested. Lyophilized crude E. multilocularis metacestode antigen, obtained from experimentally infected Meriones unguiculatus, was kindly provided by M. Liance (Laboratoire de Parasitologie-Mycologie, Hôpital Henri Mondor AP-HP, and Université Paris XII, Créteil, France).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Schagger and von Jagow (19). Briefly, antigens were solubilized in sample buffer (900 mM Tris-HCl [pH 8.45], 12% glycerol, 4% SDS, 0.005% phenol red) in the presence of 5% (vol/vol) 2-mercaptoethanol (Sigma). Antigen concentrations ranging from 25 to 500 µg per minigel (8.3 cm wide) and polyacrylamide concentrations ranging from 6 to 15% were used. Antigens were electrophoresed by using Mini-Protein III electrophoresis cells (Bio-Rad). Each gel included a set of low or high prestained molecular size standards (Bio-Rad). Antigens were transferred to nitrocellulose membranes (Schleicher & Schuell) by electroblotting (Mini Trans-Blot electrophoretic transfer cell; Bio-Rad) in transfer buffer (25 mM Tris-HCl, 193 mM glycine, 10% methanol [pH 8.3]) for 1 h at 300 mA. Blots were blocked with 0.5% casein in Tris-buffered saline (TBS) (20 mM Tris, 150 mM NaCl [pH 7.6]), washed with TBS, and then cut into 2-mm strips. Strips were incubated with 1:100 dilutions of patient sera in TBS for 45 min, washed with TBS three times, incubated with alkaline phosphatase-conjugated goat anti-human immunoglobulin G (Sigma), diluted 1:10,000 in TBS for 45 min, and washed three times with TBS. All incubations were performed at room temperature on a rotary shaker. Antibody reactivity was visualized with 5-bromo-4-chloro-3-indolylphosphate and toluidinium-nitroblue tetrazolium substrate.

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In the initial experiments, immunodominant bands were determined, and then the resolution of these bands was improved (Fig. 1). Detection of 8- to 50-kDa bands and background was remarkably reduced at low antigen concentrations. On the other hand, resolution of higher-molecular-weight bands was improved. Since serum samples from patients with AE were reactive with high-molecular-weight antigens, these bands were resolved by lowering the polyacrylamide gel concentration (Fig. 1B). The analysis was focused on the approximately 70- and 90-kDa bands, where serum samples from patients with AE exhibited strong reactivity. Recognition of bands was higher with 100 μg of antigen per minigel and 8% polyacrylamide concentration (Fig. 2). The bands showed sharper and broader characteristics and seemed to be suitable for the serodiagnosis of AE.

The presence of both 70- and 90-kDa bands (Em70 and Em90, respectively) was interpreted as a positive result for AE. Serum samples from patients with AE exhibited strong reactivity with 100% sensitivity at 70- and 90-kDa bands, and these bands were not recognized with sera from CE patients with one exception (Fig. 2). The exception was a patient with osseous hydatidosis who had undergone surgical resection and was diagnosed with CE by histopathological examination. None of the serum samples from patients with other parasitic infections and healthy individuals showed positive results (results not shown). The specificities in patients that did not have echino-

FIG. 1. Determination of immunodominant bands and improving the resolution of these bands from a set of serum samples of patients with AE (m) or CE (c). (A) Effect of lowering the antigen concentration from 500 to 25 μg per minigel. SDS-PAGE was performed with 15% polyacrylamide gels. The antigen concentrations (in micrograms per minigel) are shown below the strips. The positions of molecular mass (MW) markers (in kilodaltons) are shown to the left of the strips. (B) Effect of lowering both the polyacrylamide and antigen concentrations. The polyacrylamide concentration is shown above the strips, and the antigen concentrations (in micrograms per minigel) are shown below the strips. The positions of 90- and 70-kDa molecular mass markers are shown to the left of the strips by the arrowheads.

FIG. 2. Immunoblotting patterns of serum samples from patients with AE or CE. All serum samples from patients with AE (lanes 1 to 39) had 70- and 90-kDa bands (arrowheads). Lanes 1 to 27, serum samples from patients with AE diagnosed by histopathology; lanes 28 to 39, serum samples from patients with AE diagnosed by radiological and clinical findings; lanes 40 to 65, serum samples from patients with CE, including a osseous hydatidosis patient (lane 40). SDS-PAGE was performed with 8% polyacrylamide gels and 100 μg of antigen per minigel. For accuracy, strips were fixed in the original position as they were cut from the membrane. MW, molecular mass markers.
cocciosis and in patients with CE were 100 and 99.08%, respectively. The overall specificity of the Em70 and Em90 markers was found to be 99.51%.

Em70 and Em90 antigens were detected at antigen concentrations as low as 25 μg/gel (Fig. 1A). The low antigen concentration reduced the frequency of occurrence and intensity of the other bands and improved the detection of Em70 and Em90. Accepting the fact that a confirmatory test should be specific with simple interpretation criteria, weak reactive bands were not evaluated. This allowed us to enhance the standardization of immunoblotting in terms of reproducibility and resolution with clear-cut interpretation of the results. Indeterminate results were not encountered, and all serum samples were successfully classified by using Em70 and Em90. In this study, preparation of strips from the crude antigen, which required no additional antigen purification methods, was found to be technically rapid.

Osseous hydatidosis is reported to account for approximately 2% of all localizations. The parasite develops multiple exogenous daughter cysts that give rise to a confluent multilocular cyst (16), and this was also observed in our patient. Interestingly, this patient was thought to have AE by the microscopic appearance of resected tissue and Em70 and Em90 serology.

The diagnostic value of Em70 and Em90 antigens could not be evaluated in the early stages of AE, since the patients in our study group had detectable masses and most had undergone surgery. Epidemiological and longitudinal analyses of patients with AE could provide further information on distinguishing between active and inactive disease and in the early diagnosis of AE. Moreover, epidemiological studies may also allow identification of the performance characteristics of Em70 and Em90.

In conclusion, Em70 and Em90 antigens have high sensitivity and specificity and may be a valuable tool for the diagnosis of AE. Evaluation of these new immunoblotting markers with double-blind international studies and characterization of two antigens may result in further improvements in the serodiagnosis and immunopathology of AE.

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