Differential serodiagnosis for cystic and alveolar echinococcosis using fractions of Echinococcus granulosus cyst fluid (antigen B) and E. multilocularis protoscolex (EM18)

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Abstract. Echinococcus granulosus cyst fluid and E. multilocularis protoscolex extract were fractionated by a single step of preparative isoelectric focusing, resulting in an antigen B-rich fraction (8-kD) and an Em18-rich fraction, respectively. The usefulness of both fractions for differential serodiagnosis of cystic (CE) and alveolar (AE) echinococcosis was evaluated by a large-scale immunoblot analysis on a battery of 354 serum samples. These included 66 from AE patients originating from four different endemic areas, 173 from CE patients originating from seven different endemic areas, 71 from patients with other parasitic diseases, 15 from patients with hepatomas, and 29 from healthy individuals. In an immunoblot with the antigen B-rich fraction, 92% (158 of 173) of the CE sera as well as 79% (52 of 66) of the AE sera reacted with the 8-kD subunit. No cross-reactivity occurred with any sera from patients with cystercerosis, other parasitic diseases, or with hepatomas, or from healthy controls. In an immunoblot with the Em18-rich fraction, all but two sera from AE patients (64 of 66, 97%) recognized Em18, and only nine of 34 CE sera from China reacted with it. All other (139) CE sera from six other countries were negative as were all (115) other non-echinococcosis sera. These findings indicate that antigen B (8-kD) is not species-specific for E. granulosus but is genus-specific for Echinococcus, and that the Em18 antigen is a reliable serologic marker for species-specific differentiation of AE from CE.

Echinococcosis, one of the most lethal parasitic zoonoses, remains a public health problem of worldwide importance and has recently gained recognition in some regions as an emerging or re-emerging disease. In humans, this disease, including cystic (CE) and alveolar echinococcosis (AE), is caused by the larval stage of two species of tapeworms (Echinococcus granulosus and E. multilocularis, respectively). Differential diagnosis between the two species has significant implications for epidemiologic studies and treatment of these diseases, especially in the regions such as North America, central Europe, and China, where both species occur sympatrically. Imaging methods, including ultrasonography, computerized tomography (CT), and magnetic resonance imaging (MRI), can provide a species-specific differential diagnosis in many cases, but diagnosis may be problematic in others. Serodiagnostic tests based on antibody detection exhibit high sensitivity and reasonable specificity, and have played an important role in confirming clinical diagnosis and in epidemiologic studies. However, the use of crude somatic antigens from E. granulosus hydatid cyst fluid (HCF) or E. multilocularis metacestodes in serologic tests frequently results in problems of cross-reactions within and/or across Echinococcus. Thus, this limits their usefulness in differential serodiagnosis of CE and AE. As a result, increasing effort has been expended in identifying and purifying species-specific echinococcal antigens for application in highly sensitive assays such as ELISAs and immunoblots.

The two major antigens of E. granulosus HCF (antigen 5 and antigen B) have been extensively characterized for their diagnostic importance by a diversity of methods. Currently, it has been generally accepted that detection of antigen B, particularly its smallest subunit of 8/12 kD, offers greater specificity than detection of antigen 5, but neither antigen B nor antigen 5 are species-specific for E. granulosus. For the serodiagnosis of AE, an ELISA using an affinity-purified Em2 antigen from E. multilocularis metacestodes has been shown to be a reliable serologic test. Its diagnostic value has been improved by the combination use of the native Em2 antigen and a recombinant antigen (II/3-10) in an ELISA known as the Em2plus-ELISA, which is now commercially available Recently, an 18-kD antigen from the E. multilocularis protoscolecites (Em18) was reported by our group as a highly species-specific and sensitive antigen with potential not only for differentiation of AE from either CE or other helminth infections, but also for differentiation of active from inactive AE. In general, there was a good correlation between Em18-immunoblots and the Em2plus-ELISA, although the specificity was somewhat higher in the former. More recently, we confirmed that both Em18-immunoblots, particularly the IgG subclass Em18-immunoblot and the Em2plus-ELISA, were reasonably reliable for long-term follow-up study of patients with AE after treatment with albendazole.

This paper reports the isolation of antigen B and Em18 by preparative isoelectric focusing (IEF) and the evaluation of their usefulness by immunoblot for serologic verification and differentiation between CE and AE from some different geographic foci.

MATERIALS AND METHODS

Parasite material. The HCF was collected from fertile lung and liver cysts obtained from E. granulosus-infected
sheep in Urumqi, China. The fluid was clarified by centrifugation at 3,000 × g for 15 min at 4°C, dialyzed against 5 mM Tris-HCl (pH 7.4) for 48 hr at 4°C, and stored at −20°C. Echinococcus multilocularis metacestodes were obtained following passage in experimentally infected Wistar rats. Crude E. multilocularis protoscolex (EmPS) antigen extract was prepared as described previously.15

**Serum samples.** A total of 40 and 37 serum samples collected in Switzerland and the United States, respectively, were examined in blind tests for preliminary study. Sixty-six individual serum samples from surgically and/or parasitologically confirmed cases of AE were obtained from China (25), Japan (18), the United States (17), and Poland (6). One hundred seventy-three serum samples from surgically and parasitologically confirmed cases of CE were obtained from China (34), Jordan (36), Australia (39), Turkey (25), the United States (8), Nepal (10), and Poland (21). All of these AE and CE cases had liver lesions(s), and 234 of 239 were obtained before treatment.

Serum samples from patients with other proven parasitic diseases included cysticercosis (23), schistosomiasis (10), paragonimiasis (10), clonorchiasis (10), sparganosis (10), fascioliasis (4), and filariasis (4). All of these sera had high antibody responses to their homologous antigens as determined previously by immunoprecipitation, ELISA, or immunoblot. Twenty-nine serum samples from healthy Japanese students with no evidence of these diseases were used as controls.

This international collaborative project on echinococcosis and cysticercosis was approved by the Human Research Committee of Gifu University. Informed consent was obtained from all serum donors prior to collection of any blood specimens at all medical institutes involved in the study. All sera were shipped to Gifu University under dry ice storage.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.** Antigen preparations were resolved by SDS-PAGE using precast 4–20% gradient gels (#01-022 for two dimensions with a 6-cm width; Tefco, Tokyo, Japan) under reducing conditions. Preserved low and broad molecular markers (161-0305 and 161-0318, respectively; Bio-Rad, Hercules, CA) were used for monitoring the electrophoresis of EmPS and HCF, either crude or fractionated, respectively. Approximately 400 μg of crude antigens and 20 μg of fractionated antigens were loaded into one large sample well of a 6-cm width gel. Electrophoresis was carried out at a constant current of 25 mA for approximately 90 min and 70 min for EmPS and HCF, respectively. Transfer to Immobilon™ polyvinylidene difluoride membranes (Millipore, Bedford, MA) was carried out at 150 mA for 4 hr. Immunoblotting was carried out using human sera diluted 1:100 and a monoclonal antibody against Em18-immunoblot analysis of crude antigens of protoscoleces of E. multilocularis. Approximately 83% (35 of 42) of the sera from patients with AE and 6% (1 of 17) of the sera from patients with CE were serologically diagnosed as AE. Four of the seven negative AE sera were from inactive cases with calcified lesions. All other sera, including neurocysticercosis (0 of 8), trematodiases (0 of 5), or nematodiases (0 of 5), were negative.

Antigen B. Immunoblot analysis of crude HCF revealed a very complex profile of immunoreactive components ranging from 8 kD to > 213 kD (lane A in Figure 1). The most dominant bands included the three regularly spaced subunits of antigen B at 8, 16, and 24 kD, and the large subunit of antigen 5 at 38 kD. When crude HCF was applied to the immunoblot to detect antibodies in individual human sera, there was little difference between the recognition patterns of AE sera compared with those of CE sera, and cross-reactions were observed with sera from patients with cysticercosis or fascioliasis. Figure 1 shows an antigen B-rich fraction obtained by preparative IEF of HCF. The fraction had a pH range of 1.4–3.2 and contained the three subunits of antigen B with a predominance of 8-kD antigen and traces of 16-kD and 24-kD antigens, and no apparent contamination was observed. The 8-kD subunit was highly immunogenic and strongly recognized by sera from patients with CE, as well as sera from patients with AE. Probing with sera from patients with cysticercosis or with other parasitic diseases failed to detect any of the three subunits.
**Figure 2.** Representative immunoblots of *Echinococcus multilocularis* protoscolex crude antigens (A) and the Em18-rich fraction (B) probed with monoclonal antibody against Em16 (M) and by individual sera from patients with cystic echinococcosis (CE) (10), alveolar echinococcosis (AE) (10), nine other diseases (OD) including cysticercosis, schistosomiasis, paragonimiasis, sparganosis, clonorchiasis, fascioliasis, filariasis, hepatomas, sarcoidosis, and one healthy control. Arrowheads indicate Em18. Lane C shows CE showing the antibody response against Em18. This was an exceptional case of CE from China that recognized Em18 (see Results and Table 1). Other CE sera were from Poland, the United States, Turkey, Jordan, Australia, China, and Nepal. Values on the left are in kilodaltons (kD).

**Table 1**

| Differential serodiagnosis of cystic (CE) and alveolar echinococcosis (AE) |
|-----------------------------|-----------------------------|-----------------------------|
| Serum samples               | No. of sera       | Antigen B fraction | Em18 fraction       |
| CE                          | 173              | 158 (92%)          | 9 (5%)             |
| China                       | 34               | 32               | 9*                 |
| Australia                   | 39               | 36               | 0                  |
| Jordan                      | 36               | 34               | 0                  |
| Turkey                      | 25               | 23               | 0                  |
| Nepal                       | 10               | 9                | 0                  |
| United States               | 8                | 8                | 0                  |
| Poland                      | 21               | 16               | 0                  |
| AE                          | 66               | 52 (79%)          | 64 (97%)           |
| China                       | 25               | 20               | 25                 |
| Japan                       | 18               | 14               | 18                 |
| United States               | 17               | 13               | 16                 |
| Poland                      | 6                | 5                | 5                  |
| Others                      | 115              | 0 (0%)           | 0 (0%)             |
| Cysticercosis               | 23               | 0                | 0                  |
| Schistosomiasis             | 10               | 0                | 0                  |
| Paragonimiasis              | 10               | 0                | 0                  |
| Clonorchiasis               | 10               | 0                | 0                  |
| Sparganosis                 | 10               | 0                | 0                  |
| Fascioliasis                | 4                | 0                | 0                  |
| Filariasis                  | 4                | 0                | 0                  |
| Hepatoma                    | 15               | 0                | 0                  |
| Normal                      | 29               | 0                | 0                  |

* See Results.

**Antigen Em18.** Immunoblotting with Em crude antigen and AE/CE sera revealed numerous bands of a wide molecular range (Figure 2A). A cluster of sharply focused bands with low molecular weights of approximately 14–20 kD was commonly recognized by sera from patients with AE. The designated Em18 (arrowheads in Figure 2) is the band just above the 16-kD band detected by a monoclonal antibody against Em16.15

As shown in Figure 2B, preparative IEF of EmPS crude antigen resulted in an Em18-rich fraction with a pH of 7.6 that contained some contaminants with much higher molecular weights. The lack of reactivity with monoclonal antibody against Em16 confirmed the absence of contamination with the Em16 antigen. The Em18 band could be clearly identified by immunoblotting using the Em18-rich fraction because only the Em18 band was located in the low molecular weight zones.

**Relative sensitivity and specificity of immunoblots with Em18- and antigen B-rich fractions.** The data on serologic differentiation of CE and AE originating from different endemic areas of the world are summarized in Table 1. In the immunoblots with the antigen B-rich fraction, 92% (158 of 173) of CE sera and 79% (52 of 66) of AE sera were positive, and no cross-reactivity occurred with any sera from patients with other parasitic diseases, patients with hepatomas, or healthy controls. There was no significant difference in sensitivity when serum samples from different countries were compared. In immunoblots with the Em18-rich fraction, 97% (64 of 66) of the AE sera recognized Em18, as did nine of 34 Chinese CE sera (lane C in Figure 2). All other 139 CE sera collected from six other countries were negative. No cross-reactivity was observed with any sera from non-echinococcosis patients or from healthy controls.

**DISCUSSION**

Currently, isolation and purification of specific *Echinococcus* antigen components have been achieved largely through the application of a variety of methods, including anion exchange chromatography,21,12 affinity with protein A or monoclonal antibodies,12,22–24 hydrophobic interaction,21 and high-performance liquid chromatography.8,21,22,24 Analytical IEF has been used to identify the Em2a antigen12 and the antigen 5 subunits.24 We used preparative IEF to isolate *Echinococcus* antigens. This method is gentle, non-denaturing, and shows high resolution of proteins that differ in pI by fractions of a pH unit. The proteins separated in the Rotofor® cell can be easily recovered once they are focused. The benefits of this technique have been demonstrated in our earlier attempts to purify Em18/16 antigen28 and *Taenia solium* glycoprotein antigens.25
In the present study, a single step of preparative IEF performed with *E. granulosus* HCF and *E. multilocularis* protoscolex extract resulted in the isolation of an antigen B-rich fraction and an Em18-rich fraction, respectively. Both fractions were evaluated for their applicability in differential serodiagnosis of CE and AE by a large-scale immunoblot analysis on a battery of 354 serum samples, including 66 from AE patients originating from four different endemic areas, 173 from CE patients originating from seven different endemic areas, 71 from patients with other parasitic diseases, 15 from patients with hepatomas, and 29 from healthy individuals.

The immunoblot with the Em18-rich fraction demonstrated that all but two sera from AE patients recognized Em18, and nine of 34 CE sera from China reacted with it. All other CE sera from other six countries (139) were seronegative for Em18 antibody as were all other non-echinococcosis sera (115). The sensitivity and overall specificity were calculated to be at least 96% and 97%, respectively (including samples with equivocal results discussed herein). These results further support our previous observations that Em18 is highly sensitive and species-specific for *E. multilocularis*. The two cases of AE, one from the United States (Alaska) and the other from Poland, that were serologically negative by Em18 and Em2⁺⁺⁺-ELISA, were shown to have calcified inactive lesions only. The nine Chinese CE cases seropositive for anti-Em18 antibody originated from Sichuan, China, where both species of *Echinococcus* exist and mixed human AE and CE infections have been documented. All nine cases (four with exceptionally high and five with relatively lower titers) were also seropositive by the Em2⁺⁺⁺-ELISA. In contrast, no CE cases from Jordan and Australia, where only CE has been reported, and from Turkey and Poland, where only sporadic cases have been reported, showed positive reactions with Em18. Similarly, Wen and others reported that the 17.5-kD band in EmPS (corresponding to the Em18) reacted with the sera of three of 18 Chinese CE cases, but did not react with any of the 23 sera from CE cases from Uruguay or Libya, where human AE has not been documented. These findings appear to support our earlier assumption that at least four of the nine Chinese CE cases showing exceptionally high optical density values in the Em2⁺⁺⁺-ELISA had also been exposed to *E. multilocularis*. This assumption deserves clarification by further investigation.

Our findings are in contrast to those of Nirmalan and Craig, who recently reported that Em18 was cross-reactive with approximately 28% of the sera from CE patients from Uruguay and the United Kingdom, two countries that are non-endemic for AE. The discrepancy may be attributed to the differences in antigen preparation or electrophoresis conditions. This problem has highlighted the need to purify the Em18 antigen. In the present study, we obtained a highly purified Em18-rich fraction by preparative IEF and found that the Em18 band could be clearly recognized in immunoblots since only the Em18 band was present in the low molecular weight regions.

Because Em18 has a relatively low molecular weight, it is essential to perform SDS-PAGE under the optimal conditions, particularly when using a crude antigen preparation. Otherwise, Em18 may not be well separated from other antigen components with similar molecular weights. In the study of Nirmalan and Craig, they used a somewhat low (12%) nongradient gel, and this may account for their different results. Furthermore, it is noteworthy that Em18 appears as a sharp band on the blots. This is an important characteristic that should be considered when interpreting the immunoblot results. If there is a diffuse band located near or at the position of the 18-kD band, it is probably a band that is not from the protoscolex, but from the microvesicle, since when crude antigens were prepared from microvesicles purified by density gradient centrifugation, they showed different patterns from those of the protoscolex, with additional bands near the positions of Em18 and Em16 (Ito A and others, unpublished data). Further studies are needed to confirm if Em18 that appeared as a rather diffuse band as in the study of Nirmalan and Craig is indeed identical to the Em18 identified in our studies.

The immunoblot with the antigen B-rich fraction revealed that 92% (158 of 173) of the CE sera and 79% (52 of 66) of the AE sera were reactive with the 8-kD subunit. No cross-reactivity occurred with any sera from patients with cysticercosis or other parasitic diseases, patients with hepatoma, or from healthy controls.

The sensitivity (92%) for the 8-kD subunit obtained in our study appears to be similar to that (94%) for the 8-kD subunit reported by Shambesh and others, as well as that (90%) for the 12-kD subunit described by Leggatt and others, but higher than that (80%) for the 12-kD subunit reported by Ioppolo and others. The cross-reactions for the 8-kD subunit with AE sera (80%) demonstrated in our study is higher than those reported in other two studies (both 40%), based on these data, it seems reasonable to conclude that the smallest subunit of antigen B (8/12-kD) is not species-specific for *E. granulosus* but is shared with *E. multilocularis*. This will limit its usefulness for serologic differentiation of CE from AE.

Interestingly, in our present study, no cross-reactivity to the 8-kD subunit was observed with sera from patients with human cysticercosis. This is in contrast to the results of previous studies in which cross-reactions occurred with approximately 5% of the sera from patients with human cysticercosis. Possible explanations for this difference are that we used a highly purified antigen preparation or that we examined an insufficient number of serum samples.

The strategy we are recommending for serodiagnosis of echinococcosis based on the present results is to perform immunoblotting or an ELISA using antigen B subunits for suspected cases of CE, and Em18-immunoblotting or the Em2⁺⁺⁺-ELISA for suspected cases of AE based on image analysis.

**Financial support:** This work was supported in part by a Grant-in-Aid for International Scientific Research (Joint Research, 06044089, 07044243, 09044279) and a Grant-in-Aid for Scientific Research (B) (10480235, 10557029) from the Ministry of Education, Science, Sports and Culture of Japan, and by a Grant-in-Aid for the Control of Emerging and Re-emerging Diseases in Japan from the Ministry of Health and Welfare of Japan to Akira Ito.

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References


