Simple and reliable preparation of immunodiagnostic antigens for Taenia solium cysticercosis.

Sako Y, Itoh S, Okamoto M, Nakaya K, Ito A.
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Running title: Simple preparation of immunodiagnostic antigens for *Taenia solium* cysticercosis

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SUMMARY

Cysticercosis caused by infection with the larval stage of *Taenia solium* is an important cause of neurological disease worldwide and immunodiagnosis is important for the control and elimination of cysticercosis. In the present study, we established a simple and reliable preparation of immunodiagnostic antigens, low-molecular-weight antigens (LMWAgs), from *T. solium* cyst fluids by cation-exchange chromatography. Banding patterns of prepared LMWAgs on SDS-PAGE were different between those from Ecuador and China isolates. All cysticercosis patient sera and some echinococcosis patient sera examined recognized both LMWAgs by enzyme-linked immunosorbent assay, but none of sera from health persons became positive. There was no statistical difference in immunodiagnostic performance of LMWAgs prepared from different geographical isolates. These results indicated that this novel immunodiagnostic antigens preparation would contribute the control and prevention of cysticercosis in endemic areas, especially developing countries.

Key words: *Taenia solium*, cysticercosis, immunodiagnostic antigens, low-molecular-weight antigens, cation-exchange chromatography, simple preparation.
INTRODUCTION

The larval stage of the pork tapeworm *Taenia solium* is responsible for cysticercosis. Humans are accidentally infected with *T. solium* by ingestion of eggs excreted with the feces of individuals harboring the adult tapeworm in the intestinal tract. The larvae migrate throughout the body, invade skeletal muscle, subcutaneous tissue, or the central nervous system, the latter of which is known as neurocysticercosis, and encyst to form cysticerci. This disease is one of the reemerging zoonoses worldwide, and it is the major etiological agent of epileptic seizures in areas of endemicity, making it a major public health problem in most developing counties (Mahanty and Garcia, 2010).

Diagnosis of neurocysticercosis has been reached by clinical criteria, computed tomography (CT), nuclear magnetic resonance imaging (MRI) (Ito and Craig, 2003). The imaging techniques are useful for diagnosis but have possibility to overlooking the infection when the number of parasites is low and/or the figures are not clear or not typical. Moreover these techniques are not suitable for the diagnosis of cysticercosis in areas where it is endemic because of the high cost. Therefore, the development of an immunodiagnostic test that detects specific antibodies in either sera or cerebrospinal fluid (CSF) is necessary because of its simplicity and reliability especially in serum.

Efforts have been directed toward identification and characterization of specific antigens, and glycoproteins in cyst fluid of *T. solium* metacestodes have widely been accepted for serodignosis purposes. Parkhouse and Harrison (1987), and Tsang *et al.* (1989) have demonstrated that lentil-lectin affinity chromatography-purified Glycoproteins that showed seven bands around 15-30 kDa were
highly specific to cysticercosis on immunoblot. These glycoproteins have been shown not to be
suitable for ELISA antigens because of a cross reactivity. We have developed a simple method to
purify diagnostic antigens also known as low-molecular-weight antigens (LMWAgS) by preparative
isolectric focusing electrophoresis (IEFE) from cyst fluid available for both immunoblot and ELISA
(Ito et al. 1998). However, as this method requires sophisticated and expensive equipments it is
difficult to apply it in endemic areas. As another way, we have reported an affinity-purification
method of them using polyclonal and monoclonal antibodies (Sato et al. 2006), but this method was
lacking in general use because it needs a specific antibody. Recombinant proteins or synthetic peptide
of them are also available for immunodiagnosis purpose (Chung et al. 1999; Greene et al. 2000; Sako
et al. 2000; Hancock et al. 2003), it is, however, confined to a small number of laboratories. Therefore
a simple and low-cost method to prepare immunodiagnostic antigens, especially ELISA antigens,
must be established urgently, because it is a key point for epidemiological studies and serodiagnosis in
endemic areas. In this study, we report a novel and reliable preparation of immunodiagnostic antigens,
LMWAgS, from cyst fluids using a cation-exchange chromatography, and the evaluation of LMWAgS
as ELISA antigens.

MATERIALS AND METHODS

Parasite materials
All cysts were collected from naturally infected pigs in China and Ecuador. Fluid from *T. solium* cysts was centrifuged at 10,000xg for 30 min at 4 °C, the supernatant was recovered and kept at -80 °C until use.

**Preparation of LMWAgs from cyst fluids**

Cyst fluid from *Taenia solium* larva was dialyzed against 10 mM HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer (pH 8.0) containing 0.5 mM EDTA. After adding CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate) to the dialysate to a final concentration of 2%, it was directly loaded on a HiTrap SP XL cation-exchange column (GE healthcare, Japan) pre-equilibrated with a start buffer (10 mM HEPES buffer, pH 8.0, containing 0.5 mM EDTA) and proteins were eluted with a gradient between 0 and 1.0 M NaCl in the start buffer or with 1.0 M NaCl in the start buffer by using Fast Protein Liquid Chromatography system (ÄKTA FPLC system, GE Healthcare, Japan). Or proteins bound to cation-exchange resins were recovered by a stepwise elution with the start buffer containing 1.0 M NaCl manually. The elutant was boiled for 20 min to precipitate the contaminants, then the supernatant was collected and kept at -20 °C.

**Serum samples**

A total of 60 serum samples from patients with cysticercosis, 30 serum samples from patients with alveolar echinococcosis (AE), 25 serum samples from patients with cystic echinococcosis (CE), and
30 serum samples from healthy persons were examined. Each diagnosis of cysticercosis, AE and CE had been carried out by imaging techniques, clinical findings, histological observations (if feasible) and/or serology. All cysticercosis patient sera were confirmed to be seropositive to LMWAgs purified by IFE that has already been reported to be reliable (Ito et al. 1998). Both AE and CE patient sera showed strong reaction to each immunodiagnostic antigens, recombinant Em18 (Sako et al. 2002) and recombinant Antigen B (Mamuti et al. 2004), respectively.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis**

Proteins were treated with a SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.0% SDS, 50 mM dithiothreitol and 10.0% glycerol) at 100 °C for 5 min and separated in a 12.5 or 15.0 % polyacrylamide gel. For immunoblot analysis, the separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane sheet (Millipore). The sheet was blocked with blocking solution (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.0% casein, 0.1% Tween 20) and probed with LMWAgs-immunized rabbit sera or cysticercosis-patient sera followed by alkaline phosphatase-conjugated anti-rabbit IgG antibody (Novagen, USA) or peroxidase-conjugated recombinant protein G (Invitrogen, USA). Nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (KPL, USA) for alkaline phosphatase and 3,3',5,5'-tetramethyl-benzidine (KPL, USA) for horseradish peroxidase were used for color development.
**Enzyme-linked immunosorbent assay (ELISA)**

ELISA plates (Nunc-ImmunoTM plate MaxiSorpTM Surface, Nalge Nunc International, Japan) were coated with 100 μl of 1 μg/ml LMWAg in PBS overnight at 4°C. The wells were blocked with 300 μl of blocking solution (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.0% casein, 0.1% Tween 20) at 37°C for 1–2 hr. After the wells were rinsed twice with PBS containing 0.1% Tween 20 (PBST), 100 μl of serum samples diluted 1:100 in blocking solution were added and incubated at 37°C for 1 hr. The wells were washed five times with PBST, incubated with 100 μl of recombinant protein G conjugated with peroxidase (Invitrogen) at 37°C for 1 hr, washed five times with PBST and then rinsed PBS once. After incubation with 100 μl of substrate (0.4 μM 2,2’-azino-di-[3-ethyl-benzthiazoline sulfonate] in 0.2 M citric acid buffer, pH 4.7) for 30 min at room temperature, the absorbance at 405 nm of each well was determined using ELISA plate reader (Immuno Mini NJ-2300, Biotec, Japan). ROC curve analyses with ELISA data of sera from cysticercosis patients and healthy persons were performed to determine optimal cutoff values.

**Statistical analysis**

The data of diagnostic performances obtained were tested by the Cochran’s Q test and post hoc analyses were conducted with McNemar’s test.
RESULTS AND DISCUSSION

Preparation of LMWAgs

Because immunodiagnostic LMWAgs in the cyst fluid have a pI value of around 9.4 (Ito et al. 1999), we performed a cation-exchange chromatography (CEC) under pH 8.0 to purify them and a single sharp peak was resolved (Fig. 1A). Immunoblot analyses with sera from cysticercosis patients and from a rabbit immunized with LMWAgs revealed that resin-bound fractions (fractions 2-5) included LMWAgs (data not shown). There were no differences in between chromatographic profiles obtained from cyst fluids from Ecuador and China isolates (data not shown). By a single chromatographic procedure we could prepare LMWAgs sufficiently, but some contaminations with high molecular size were sometimes observed although it depended on the batch of cyst fluid used for preparation (Fig. 1B). From the fact that Echinococcus Antigen B family to which LMWAgs belong is thermostable (Oriol et al. 1971), we speculated that LMWAgs also had the thermostability and that, if the contaminated proteins were non-heat-resistance, the contaminants could be removed by heat-denatured precipitation and LMWAgs would be recovered in the supernatant. As expected, the heat-treatment precipitated contaminated proteins but not LMWAgs (Fig. 1B). The CEC-prepared LMWAgs were migrated as a broad band between 10 to 25 kDa (Fig. 1C). There were critical differences in banding pattern between those from Ecuador and China isolates. Previously, we have demonstrated that: (1) T. solium could be divided into 2 genotypes, African/American and Asian, on the basis of mitochondrial DNA analyses (Nakao et al. 2002); (2) each LMWAgs purified from cyst...
fluids of two genotypes by antibody-affinity chromatography showed differences in banding patterns (Sato et al. 2006); (3) such differences were disappeared by glycan removal with PNGase F (Sato et al. 2006). In other words, glycoforms with different masses or a different number of \( N \)-linked oligosaccharides are the putative cause of the different banding pattern of Glycoproteins from Asian, African, or American geographical origin. Therefore, the difference in banding pattern of the CEC-prepared LMWAg between two genotypes might be caused by post translational modification. Further analysis on post translational modification of the CEC-prepared LMWAg has to be performed, in addition to confirmation of producibility of preparation by using cyst fluids from several geographical different isolates.

Evaluation of the CEC-prepared LMWAg by ELISA

Evaluations of the CEC-prepared LMWAg as ELISA antigens were carried out with serum samples from 60 cysticercosis patients, 30 alveolar echinococcosis patients, 30 cystic echinococcosis patients, and 41 healthy persons (Fig. 2. and Table 1). When crude CF antigens were used, more than half of echinococcosis patient sera and one health person serum resulted in seropositive and one cysticercosis case was negative. In contrast, all sera from cysticercosis cases showed positive reactions to both the CEC-prepared LMWAg of Ecuador- and China-isolates, and some sera from alveolar and cystic echinococcosis patients showed weak cross reactions, almost close to the cut-off borderline, to both antigens. The overall specificities of crude CF antigen and the CEC-prepared LMWAg from Ecuador
and China isolates were 66.3%, 92.1% and 93.1%, respectively. There was no statistically significant
difference in immunodiagnostic performance between the CEC-prepared LMWAgs from Ecuador and
China isolates. In this study, we used only cysticercosis patient sera positive to IEFE-purified
LMWAgs and echinococcosis patient sera with strong reactions to each homologous
immunodiagnostic antigen for evaluation, which indicated sera used were under some biases and
sensitivities and specificities based on the result of ELISA did not reflect real cysticercosis and
echinococcosis patient populations. Therefore, we need further evaluation with more sera from
cysticercosis and echinococcosis patients, and also patients with other parasitic infections to reveal
immunodiagnostic performance quality of the CEC-prepared LMWAgs.

We previously have reported that the origin of the fluid from *T. solium* cysts may have affected the
antigenicity of LMWAgs (Sato et al. 2006). As mentioned earlier, the mitochondrial analyses revealed
that *T. solium* is able to be divided into two genotypes, African/American and Asian genotypes (Nakao
*et al.* 2002). Furthermore, the purified LMWAgs could be differentiated two banding patterns
corresponded to two genotypes. Although there were no differences in diagnostic sensitivities between
LMWAgs from two genotypes, reactivities of sera from American patients to African/American
LMWAgs were higher than those of Asian patients, and the inverse phenomenon was observed (Sato
*et al.* 2003). This suggested that immunodiagnostic test in each endemic area should be performed by
using LMWAgs prepared from *T. solium* cysts collected in each own endemic area. Therefore,
establishment of a useful preparation of LMWAgs capable to easily apply in endemic area is urgently
required. Recently, a simple enrichment of 120- and 150-kDa immunodiagnostic antigen complexes of *T. solium* cyst fluids by trichloroacetic acid/acetone mixture precipitation has been reported (Lee et al. 2010). The antigens purified this method showed immunodiagnostic sensitivity and specificity of 97.7% and 98.7% against active neurocysticercosis. However, this method requires the hazardous reagent, which suggested that it was difficult to apply in general. In contrast, the CEC method does not need such reagent and, moreover, can be performed with a syringe manually without expensive equipments (data not shown). This feature would make easy to apply the CEC method in endemic area, especially in developing counties.

The CEC-prepared LMWAgs could be utilized to detect *T. solium* infections in pigs by immunoblot analysis and ELISA (data not shown). For this purpose, we need to consider the cross reaction with sera from pigs infected with other infective agents, especially *Taenia hydatigena*. Previously, no cross reactions to LMWAgs have been demonstrated in pigs infected with *T. hydatigena* by ELISA (Sato et al. 2003). However, the number of serum examined seems to be not enough to conclude that there were no cross-reactions. Therefore, a large scale evaluation in pigs must also be performed.

Interestingly, the CEC method is applicable to purify LMWAgs family proteins from *Taenia saginata*- and *Taenia asiatica*-cyst fluids (data not shown). And also, ELISA using sera from bovines experimentally infected with *T. saginata* and LMWAgs antigens prepared from *T. saginata* cyst fluids revealed that those antigens have high values to detect *T. saginata* infections with dramatic decreasing of background absorbance values obtained using crude cyst fluids (our unpublished data). More
detailed characterization of the CEC-prepared LMWAgs from \textit{T. saginata} and \textit{T. asiatica} cyst fluids must be performed using more sera from animals with cysticercosis and other parasitic infections.

In conclusion, we have established a simple and reliable preparation of LMWAgs with high immunodiagnostic performance from \textit{T. solium} cyst fluids by the combination of the CEC and heat-treatment. The CEC-prepared LMWAgs is highly specific to cysticercosis, but analyses on the B-cell epitopes must be carried out to eliminate cross-reactivity with echinococcosis patient sera. The preparation method established would contribute to the successful control of cysticercosis in endemic areas.

ACKNOWLEDGEMENTS

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FINANCIAL SUPPORT

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Table 1. Results of ELISA using crude CF from Ecuador isolate, LMWAgs from Ecuador isolate and LMWAgs from China isolate

<table>
<thead>
<tr>
<th>Disease category</th>
<th>No. of exam</th>
<th>No. of positive (%)</th>
<th>Mean absorbance ± SD (Range: max-min)</th>
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<tr>
<td></td>
<td></td>
<td>crude CF Ecuador</td>
<td>LMWAgs Ecuador</td>
</tr>
<tr>
<td>Cysticercosis</td>
<td>60</td>
<td>59 (98.3)</td>
<td>60 (100)</td>
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<tr>
<td>Alveolar Echinococcosis</td>
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<tr>
<td>Healthy persons</td>
<td>41</td>
<td>1 (2.4)</td>
<td>0</td>
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Fig. 1. Preparation of LMWAgs from *T. solium* cyst fluid. (A) Chromatographic profile of *T. solium* cyst fluid from Ecuador isolate by FPLC system. Plain line, absorbance at 280 nm; dash line, linear gradient of NaCl. (B) Each fraction by a cation-exchange chromatography, and the supernatant and the precipitate after heat-treatment were subjected to 12.5% SDS-PAGE and stained with Coomassie blue. Lane 1, crude CF; lane 2, fraction 2 from the CEC; lane 3, fraction 3 from the CEC, lane 4, fraction 4 from the CEC; lane 5, the supernatant after heat-denatured treatment; lane 6, the precipitate after heat-denatured treatment. The contaminants part detected depending on the batch of cyst fluids is indicated by an asterisk. (C) 15.0% SDSPAGE analysis of LMWAgs prepared from Ecuador and China isolates.

Fig. 2. Evaluations of three different antigens, crude CF from Ecuador isolate, LMWAgs from Ecuador isolate and LMWAgs from China isolate by ELISA. Sere from 60 patients with cysticercosis (red closed circle), 30 with alveolar echinococcosis (blue closed circle), 30 with cystic echinococcosis (green closed circle), and 41 healthy people (black closed circle) were used. The cut-off values for crude CF, LMWAgs from Ecuador, and LMWAgs from China isolate are 0.310, 0.075, and 0.085, respectively, and are indicated by the dashed lines.
Table 1. Results of ELISA using crude CF from Ecuador isolate, LMWAgs from Ecuador isolate and LMWAgs from China isolate

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