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

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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 a 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.

19 INTRODUCTION

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

27 Diagnosis of neurocysticercosis has been reached by clinical criteria, computed tomography (CT),
28 nuclear magnetic resonance imaging (MRI) (Ito and Craig, 2003). The imaging techniques are useful
29 for diagnosis but have possibility to overlooking the infection when the number of parasites is low
30 and/or the figures are not clear or not typical. Moreover these techniques are not suitable for the
31 diagnosis of cysticercosis in areas where it is endemic because of the high cost. Therefore, the
32 development of an immunodiagnostic test that detects specific antibodies in either sera or
33 cerebrospinal fluid (CSF) is necessary because of its simplicity and reliability especially in serum.
34 Efforts have been directed toward identification and characterization of specific antigens, and
35 glycoproteins in cyst fluid of *T. solium* metacestodes have widely been accepted for serodignosis
36 purposes. Parkhouse and Harrison (1987), and Tsang *et al.* (1989) have demonstrated that lentil-lectin
37 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 isoelectric 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 IEFE 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-benzidene (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 LMWAgs 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 1hr, 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 form 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 LMWAgs between two genotypes might be caused by post translational modification. Further analysis on post translational modification of the CEC-prepared LMWAgs 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 LMWAgs by ELISA

Evaluations of the CEC-prepared LMWAgs 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 LMWAgs 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 LMWAgs 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

171 required. Recently, a simple enrichment of 120- and 150-kDa immunodiagnostic antigen complexes
172 of *T. solium* cyst fluids by trichroroacetic acid/acetone mixture precipitation has been reported (Lee *et*
173 *al.* 2010). The antigens purified this method showed immunodiagnostic sensitivity and specificity of
174 97.7% and 98.7% against active neurocysticercosis. However, this method requires the hazardous
175 reagent, which suggested that it was difficult to apply in general. In contrast, the CEC method does
176 not need such reagent and, moreover, can be performed with a syringe manually without expensive
177 equipments (data not shown). This feature would make easy to apply the CEC method in endemic
178 area, especially in developing counties.
179 The CEC-prepared LMWAgs could be utilized to detect *T. solium* infections in pigs by immunoblot
180 analysis and ELISA (data not shown). For this purpose, we need to consider the cross reaction with
181 sera from pigs infected with other infective agents, especially *Taenia hydatigena*. Previously, no cross
182 reactions to LMWAgs have been demonstrated in pigs infected with *T. hydatigena* by ELISA (Sato *et*
183 *al.* 2003). However, the number of serum examined seems to be not enough to conclude that there
184 were no cross-reactions. Therefore, a large scale evaluation in pigs must also be performed.
185 Interestingly, the CEC method is applicable to purify LMWAgs family proteins from *Taenia*
186 *saginata*- and *Taenia asiatica*-cyst fluids (data not shown). And also, ELISA using sera from bovines
187 experimentally infected with *T. saginata* and LMWAgs antigens prepared from *T. saginata* cyst fluids
188 revealed that those antigens have high values to detect *T. saginata* infections with dramatic decreasing
189 of background absorbance values obtained using crude cyst fluids (our unpublished data). More

detailed characterization of the CEC-prepared LMWAgs from *T. saginata* and *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 *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.

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268

269

Table

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

Disease category	No. of exam	No. of positive (%)			Mean absorbance ± SD (Range: max-min)		
		crude CF Ecuador	LMWAgs Ecuador	LMWAgs China	crude CF Ecuador	LMWAgs Ecuador	LMWAgs China
Cysticercosis	60	59 (98.3)	60 (100)	60 (100)	1.202 ± 0.517 (2.030-0.140)	1.240 ± 0.555 (2.07-0.100)	1.177 ± 0.520 (2.090-0.110)
Alveolar Echinococcosis	30	15 (50.0)	4 (13.3)	4 (13.3)	0.434 ± 0.347 (1.190-0.010)	0.040 ± 0.033 (0.120-0.010)	0.051 ± 0.070 (0.340-0.000)
Cystic Echinococcosis	30	18 (60.0)	4 (13.3)	3 (10.0)	0.511 ± 0.433 (1.680-0.020)	0.045 ± 0.046 (0.220-0.010)	0.043 ± 0.038 (0.210-0.010)
Healthy persons	41	1 (2.4)	0	0	0.059 ± 0.072 (0.340-0.010)	0.010 ± 0.011 (0.050-0.000)	0.012 ± 0.012 (0.060-0.000)

278 Figure legends

279 Fig. 1. Preparation of LMWAgs from *T. solium* cyst fluid. (A) Chromatographic profile of *T. solium*
280 cyst fluid from Ecuador isolate by FPLC system. Plain line, absorbance at 280 nm; dash line, linear
281 gradient of NaCl. (B) Each fraction by a cation-exchange chromatography, and the supernatant and
282 the precipitate after heat-treatment were subjected to 12.5% SDS-PAGE and stained with Coomassie
283 blue. Lane 1, crude CF; lane 2, fraction 2 from the CEC; lane 3, fraction 3 from the CEC, lane 4,
284 fraction 4 from the CEC; lane 5, the supernatant after heat-denatured treatment; lane 6, the precipitate
285 after heat-denatured treatment. The contaminants part detected depending on the batch of cyst fluids is
286 indicated by an asterisk. (C) 15.0% SDSPAGE analysis of LMWAgs prepared from Ecuador and
287 China isolates.

288

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

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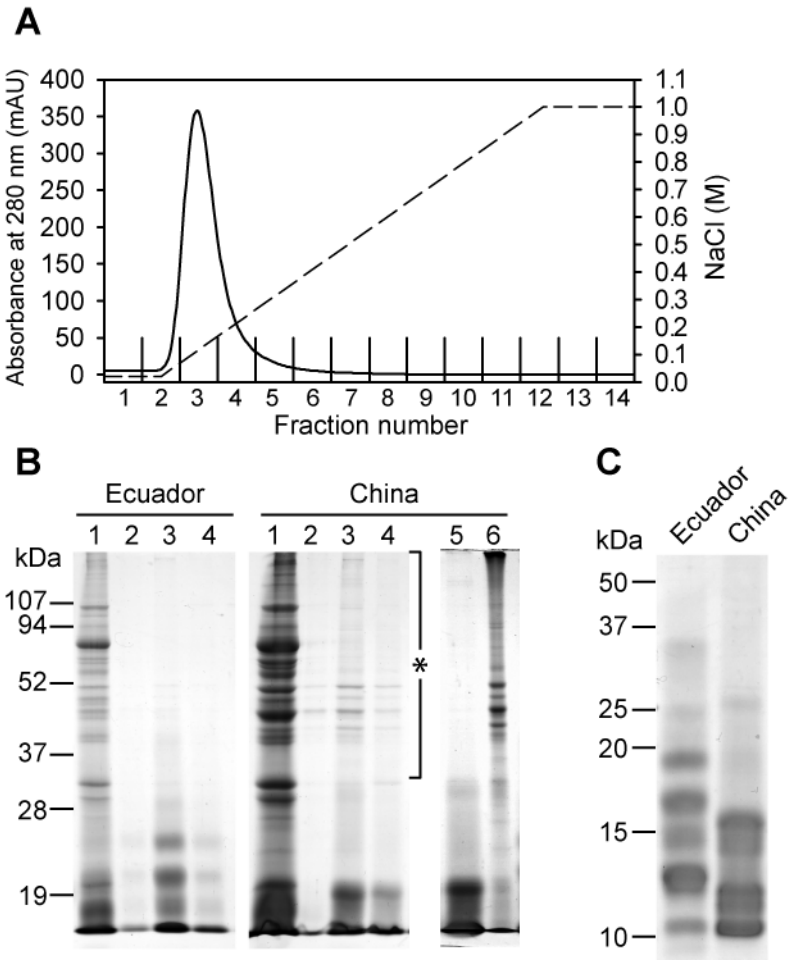
Healthy persons	41	1 (2.4)	0	0	0.059 ± 0.072 (0.340-0.010)	0.010 ± 0.011 (0.050-0.000)	0.012 ± 0.012 (0.060-0.000)


A
Absorbance at 280 nm (mAU)
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1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fraction number
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25
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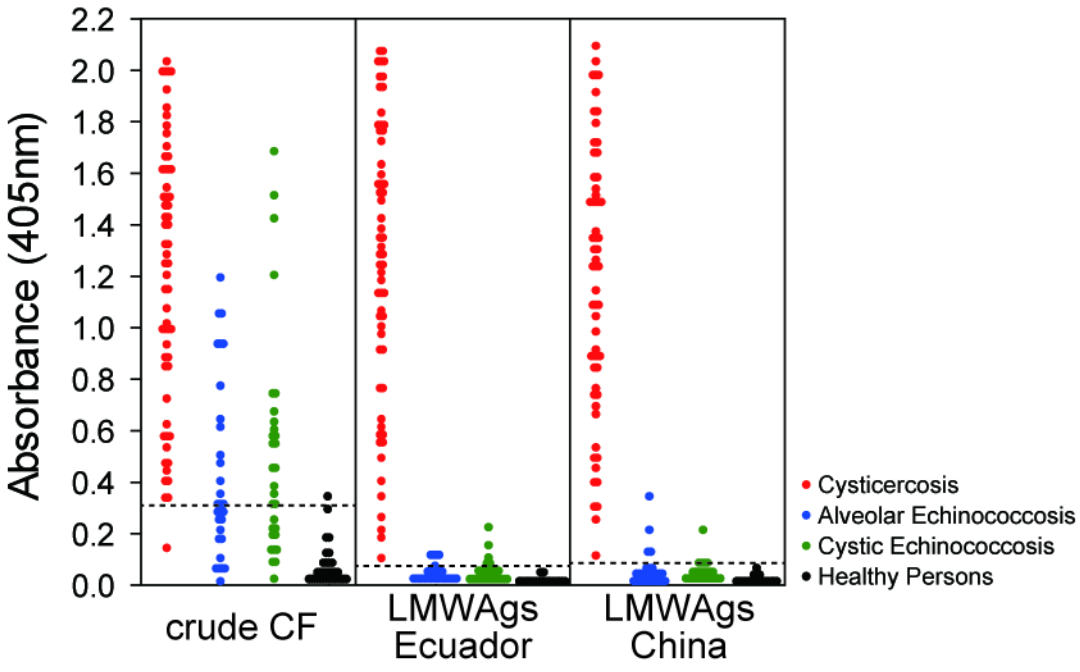
Absorbance (405nm)
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crude CF
LMWAgs Ecuador
LMWAgs China
Cysticercosis
Alveolar Echinococcosis
Cystic Echinococcosis
Healthy Persons