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ORIGINAL PAPER

Differential diagnosis of cystic and alveolar echinococcosis using an immunochromatographic test based on the detection of specific antibodies

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Abstract Human cystic and alveolar echinococcoses are zoonotic diseases caused by the larval stages of *Echinococcus granulosus* and *Echinococcus multilocularis*, respectively. As the diseases are co-endemic in many areas of the world, a simple and rapid test for the differential diagnosis of cystic echinococcosis (CE) and alveolar echinocoocosis (AE) is needed. Here, we describe the development of an immunochromatographic test (ICT) using crude hydatid cyst fluid and a recombinant 18-kDa protein (rEm18) as antigens for the detection of *E. granulosus* and *E. multilocularis* antibodies in serum samples. The ICT was evaluated with serum samples from 195 echinococcosis patients from different endemic areas in northwestern China. These included 144 from CE patients, 51 from AE patients, 67 from patients with other parasitic diseases, 13 from patients with serous hepatic cysts, and 60 from healthy individuals. The sensitivity and specificity of the ICT for CE were 91.0 and 96.9 % and for AE were 98.0 and 99.3 % with diagnostic efficiencies of 94.1 and 99.1 %, respectively. No significant differences and high degrees of agreement were found between the ICT and an enzyme-linked immunosorbent assay for both CE and AE. Five serum samples from cysticercosis patients and one serum sample from a healthy control were found positive for CE with the ICT. These findings indicate that this test allows for discrimination between both forms of human echinococcosis. In conclusion, the ICT developed in this study is a promising tool for the simultaneous detection and discrimination of CE and AE. This test will be useful for serodiagnosis of CE and AE in clinical settings and screening programs.

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Introduction

Human echinococcosis is a widespread and potentially lethal parasitic zoonosis caused by larval stages of cestodes belonging to the genus *Echinococcus* (family Taeniidae). Cystic echinococcosis (CE) and alveolar echinococcosis (AE), caused by infection with the larval stages of *Echinococcus granulosus* and *Echinococcus multilocularis*, respectively, are the clinically and epidemiologically most important forms of echinococcosis and are among the world's most dangerous zoonoses (Pawłowski et al. 2001; McManus et al. 2003). *E. granulosus* has a worldwide distribution whereas *E. multilocularis* is mainly distributed in northern hemisphere countries in the higher latitudes and is recognized as an emerging and re-emerging zoonosis (McManus et al. 2003; Craig 2003). The diseases are co-endemic in large areas of the world, including the northwest part of China (Craig et al. 1992; Ito et al. 2003; McManus et al. 2003; Yang et al. 2010; Torgerson et al. 2010). Larval infection is characterized by long-term growth of metacestodes in the intermediate host. CE and AE are distinguished by the development of unilocular fluid-filled bladders (also known as hydatid cysts) and multilocular root-like network of interconnecting vesicotubular structures, respectively, in internal organs (mainly liver). In most cases, the early stages of infection are asymptomatic, even up to 10 or more years after initial parasite infection, so early diagnosis and treatment of the diseases, especially

during the asymptomatic period, are important for reduction of morbidity and mortality, since pathogenicity is high and prognosis for patients with echinococcosis, especially AE, is often poor (Craig 2003).

At present, diagnosis of CE and AE is primarily based on imaging techniques, such as radiology, ultrasonography, computed axial tomography (CT scanning), and magnetic resonance imaging (Zhang and McManus 2006). However, these physical imaging techniques are sometimes limited by the small size of lesions to be visualized and by atypical images, which are difficult to distinguish from abscesses or neoplasms. Moreover, some of these imaging techniques are unsuitable for diagnosis in remote areas and isolated communities. Diagnostic methods that are relatively easy to use and that are cheap are required. Therefore, immunological tests have been considered to be useful methods for confirming clinical findings in the case of unclear images for obtaining information on the parasite in question or for surveying endemic areas where imaging techniques are not readily available (Gottstein 1992; Ito et al. 2007). Serology has a very long history and almost all serological tests, such as indirect hemagglutination or latex agglutination, immunoelectrophoresis, immunoblotting, complement fixation, immunoenzymatic tests, and indirect fluorescent antibody tests (Zhang and McManus 2006), have been used in the diagnosis of human CE and/or AE. In the past decades, major advances have been achieved in the purification, cloning, and characterization of relevant *E. granulosus* antigens. Several reports on the diagnostic evaluation of native antigens, such as *E. granulosus* hydatid cyst fluid (HCF), purified components from HCF and *E. granulosus* adult-worm antigen, and recombinant *E. granulosus* antigens, are available (Ersfeld et al. 1997; Carmena et al. 2006). HCF is considered the main antigen source for immunodiagnosis of human CE and crude HCF has been shown to have a higher sensitivity (up to 95 %) than purified HCF components (Zhang et al. 2003). An 18-kDa antigen from *E. multilocularis* (Em18) was cloned and evaluated in immunological tests, such as enzyme-linked immunosorbent assay (ELISA) and immunoblot analysis. Em18 was found to be a highly species-specific antigen with the potential not only to differentiate between AE and CE or other helminth infestations but also to distinguish between an active and inactive AE (Ito et al. 2002; Sako et al. 2002; Xiao et al. 2003; Zhang et al. 2003). However, these methods are time consuming and require special materials and equipment, which make them laborious for clinical applications. In contrast, dipstick tests (DSTs) and immunochromatographic tests (ICTs) are simple, rapid, and reliable methods for the detection of pathogen-specific antibodies. Previously, a DST and an ICT were developed for the diagnosis of CE and AE using HCF and rEm18, respectively (Al-Sherbiny et al. 2004; Sako et al. 2009). In the present study, we designed an ICT using HCF and rEm18 for the differential diagnosis of CE and AE and compared the test with an ELISA.

Materials and methods

Ethics statement

This study was reviewed and approved by the Ethics Review Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention in Shanghai. All subjects gave their informed written consent specifying that their serum samples can be used for future studies. All serum samples were given a unique identification number to ensure that they were anonymized for any subsequent study. For the origin of the serum samples see next section.

Serum samples

One hundred forty-four serum samples from CE patients (69 males and 75 females ranging from 15–81 years of age) were kindly provided by the Qinghai Provincial Center for Disease Control and Prevention (n=43), the Gansu Provincial Center for Disease Control and Prevention (n=57), and the Center for Disease Control and Prevention of Xinjiang Uygur Autonomous Region (n=44; 135 serum samples were from patients with cysts in the liver, 7 from patients with cysts in the lungs, and 2 from patients with cysts in the liver and lungs). Fifty-one serum samples from patients with hepatic AE (29 males and 22 females ranging from 21–55 years of age) were kindly provided by the Center for Disease Control and Prevention of Ningxia Hui Autonomous Region (n=34) and the Gansu Provincial Center for Disease Control and Prevention (n=17). Before samples were collected, the medical history of patients was taken to make sure that the patients have not been previously diagnosed with CE/AE and treated for echinococcosis. CE and AE patients were diagnosed on clinical and imaging findings or surgery according to the expert consensus for the diagnosis and treatment of CE and AE in humans (Brunetti et al. 2010). All samples were collected before treatment, i.e., before initiation of any chemotherapy. According to the criteria for classification of ultrasound images of CE, among the 144 CE cases, 1 had a CL cyst, 15 had CE1 cysts, 54 had CE2 cysts, 67 had CE3 cysts, and 7 had CE4 or CE5 cysts (Li et al. 2010). Of the 51 AE cases, 2 were classified as AE1, 8 as AE2, and 16 as AE3 and 25 were grouped as AEf including AE2f and AE3f (Li et al. 2010).

In addition, 13 serum samples from patients with serous hepatic cysts and 67 serum samples from patients with other parasitic diseases, including 25 with cysticercosis, 15 with schistosomiasis (*Schistosoma japonicum*), 10 with toxoplasmosis, 8 with paragonimiasis, and 9 with clonorchiasis as well as serum samples from 60 healthy donors were included as negative controls.

Antigens preparation

HCF of *E. granulosus* (G1 genotype) was collected from fertile sheep liver cysts obtained from Emin County (Xinjiang Uygur Autonomous, China). HCF was centrifuged at 3,000×*g* for 30 min to remove protoscoleces and large particles, and the supernatant was stored at −80 °C until use. HCF fraction was prepared according to Oriol et al. (1971). Briefly, concentrated HCF was dialyzed against 5 mM acetate buffer, pH 5 for 24 h. The resulting precipitate was solubilized in 0.2 M phosphate buffer, pH 8, and host globulins were removed by salting out with ammonium sulfate at 40 % saturation.

Cloning, expression, and purification of rEm18 were performed as described by Sako et al. (2002) with some modifications. Briefly, total RNA was prepared from *E. multilocularis* protoscoleces isolated from alveolar hydatid cysts developed in the liver of BALB/c mice (Gao et al. 2012a). Full-length cDNA of Em18 was synthesized by reverse transcription-polymerase chain reaction using primers containing *BamH I* (forward) and *EcoR I* (reverse) restriction sites added to 5′ end to facilitate subsequent cloning of the PCR products. The primers were 5′-GCGGATCCAAGGAGTCTGACTTAGCGGA-3′ (forward primer) and 5′-GCGAATTCTTTGAGGTTGGCCAGCTTC3′ (reverse primer; restriction sites underlined). PCR products were sequenced and cloned into pGEX-3X vector (Amersham Pharmacia Biotech, Uppsala, Sweden) for expression in *Escherichia coli* BL21 cells. The recombinant antigen was expressed as a fusion protein with glutathione S-transferase and purified by glutathione Sepharose 4B (Amersham Pharmacia Biotech) using the batch method following the manufacturer's instruction.

Preparation of immunochromatographic strips and test

Purified HCF (2 mg/ml), rEm18 (1 mg/ml), and chicken anti-protein G antibody (1 mg/ml) were sprayed onto nitrocellulose membranes in a 1-mm-wide line (0.8 μg/cm) as test lines (test 1 (HCF antigen) and test 2 (rEm18)) and control line (chicken anti-protein G antibody), respectively. Detecting reagent (protein G-colloidal gold conjugate) was incorporated into the sample pad. The test was carried out as follows: 10 μl of serum or 20 μl of whole blood (collected in the presence heparin (15 U/ml) as anticoagulant) was applied into the sample pad of the strip, then two drops of phosphate-buffered saline (PBS) were added, and the test was evaluated after 15 min. The samples were judge as positive once pink color appeared in the test line(s) regardless of weak or strong color.

Enzyme-linked immunosorbent assay

The ELISA was performed essentially as described by Lorenzo et al. (2005). Briefly, microtitration plates (Corning Inc. USA) were coated with 0.3 μg of rEm18 antigen per well or with 2 μg of HCF antigen per well diluted in 100 μl of coating buffer (50 mM carbonate/bicarbonate buffer; 1.5 g Na_2CO_3 and 2.9 g $NaHCO_3$ dissolved in 1,000 ml H_2O, pH 9.6) and incubated overnight at 4 °C. After the coating solution was discarded, each well was blocked with 100 μl of 5 % non-fat milk powder in PBS for 1 h at 37 °C and washed with PBS/0.05 % Tween 20 (PBS-T). Then, 100 μl serum samples diluted 1:100 in PBS-T were pipetted into the wells. After 90 min of incubation at 37 °C, the plates were washed three times with PBS-T. Then, 100 μl of peroxidase conjugated goat anti-human immunoglobulin G (Invitrogen Corp., Carlsbad, CA, USA; ZYMED) diluted 1:30,000 was dispensed into each well and incubated for 1 h at 37 °C. After washing, 100 μl of 3,3′,5,5′-tetramethylbenzidine (Tiangen Biotech Co., LTD) was added into each well and incubated for 5 min at room temperature with shaking. The reaction was stopped by adding 100 μl of 1.5 M H_2SO_4. Optical densities at 450 nm were read in an automatic microELISA reader (680XR, Bio-RAD). The mean absorbance values of the 60 healthy donors plus 3 standard deviations were used to establish a cutoff value. All serum samples were analyzed in duplicate. If the mean value was higher than the cutoff value, the sample was considered positive for anti-hydatid antibodies.

Data analysis

The definitions for sensitivity, specificity, true-positive values, false-positive values, true-negative values, false-negative values, and diagnostic efficiency were those as described by González-Sapienza et al. (2000). Sensitivity, specificity, true-positive values, false-positive values, true-negative values and false-negative values were calculated from a 2×2 table. Chi-square test was used for comparing the diagnostic parameters of the different tests. A P value greater than 0.05 was considered to be statistically significant. Kappa analysis was used to estimate the degrees of agreement between ICT and ELISA. A κ value of 0.75–1.00 represents an "excellent agreement".

Results

Figure 1 shows examples for the reactivity of the ICT with different serum samples. With negative serum samples, only the control line (chicken anti-protein G antibody) developed (Fig. 1a). When testing serum samples from patients with CE, the control line and test line 1 (HCF antigen) turned pink (Fig. 1b). With serum samples from patients with AE, all three lines appeared pink (Fig. 1c). Occasionally, when testing serum samples from AE patients, only the control line and test line 2 (rEm18 antigen) developed (Fig. 1c). If this occurred, the serum sample was still considered as positive for

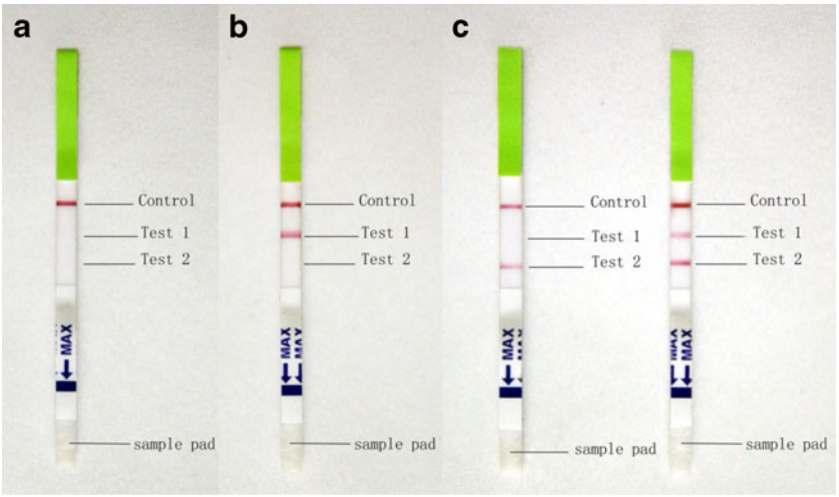

Fig. 1 Example of ICT strips tested with serum samples from patients with cystic and alveolar echinococcosis. **a** Result with a negative serum sample: only the control line turned *pink*. **b** Result with a serum sample from a patient with cystic echinococcosis (CE): the control line and test line 1 turned *pink*. **c** Results with serum samples from two patients with alveolar echinococcosis (AE): either the control line and test line 2 (left ICT strip) or all three lines (right ICT strip) turned *pink*

AE. In the case that the control line did not appear, the test was regarded as invalid even if test lines 1 and/or 2 turned pink.

Of the 144 CE serum samples tested with the ICT method, 131 were found to be positive for CE while 2 were also revealed to be positive for AE. Similar results were also obtained with the ELISA methods: 133 positive for CE and 4 positive for AE (Table 1). However, patients with CE4/CE5 cysts exhibited lower activities than those with CE1, CE2, or CE3 cysts (Table 1). Only 43 % (3/7) and 57 % (4/7) of serum samples from patients with CE4/CE5 cysts gave positive reaction with the ICT and ELISA method, respectively. Also, patients with pulmonary CE showed lower reactivity: only 57 % (4/7) of serum samples from patients with cysts in the lungs were positive in both tests (Table 1). Of the 51 AE serum samples, both ICT and ELISA methods recognized 50 positive for AE (Table 1). Only one serum sample from a patient with an AE1 cyst (early-stage disease) did not show a positive reaction with both methods (Table 1). Of the 50 positive AE serum samples, 41 and 36 were also positive for CE in the ELISA and ICT, respectively. This result indicates that the ICT method is slightly more discriminating with respect to detection of AE positive serum sample based on the reaction with the HCF antigen. Of the 80 serum samples from patients with other parasitic disease or hepatic cysts, five and nine were found to be positive for CE with the ICT and ELISA methods, respectively, and none to be positive for AE with either method (Table 1). Interestingly, almost all of the positive serum samples were from cysticercosis patients who were infested with the larval stage of another cestode, *Taenia solium* (pork tapeworm). This finding suggests that there is some cross-reactivity between CE and cysticercosis with respect to the HCF antigen. However, this cross-reactivity was somewhat lower with the ITC (5 out of 25) compared to the ELISA (8 out of 25). Finally, of the 60 serum samples from healthy donors, only one was positive for CE with the ICT methods while two were positive for CE and one for AE with the ELISA method (Table 1).

In Table 2, the diagnostic parameters of the ELISA and ICT methods for the detection of CE and AE are listed. For both methods, the sensitivity and specificity for diagnosis of CE were between 91 and 97 % while those for AE were slightly higher (≥98 %). For the diagnostic efficiency, not much difference was found between the two techniques except that the diagnostic efficiency for the detection of AE was slightly higher (>98 %) than that of CE (93–94 %). In conclusion, all diagnostic parameters for both methods for the detection of both CE and AE were very similar and greater than 90 %. Statistical analyses revealed that there were no significant differences between the diagnostic parameters of the ELISA and ICT techniques ($P>0.5$, χ^2 test). The only marked difference was that the performance of the test for AE was slightly better than the performance of the test for CE. In addition, high degrees of agreement were observed between ICT and ELISA for the detection of both CE ($\kappa=0.91$) and AE ($\kappa=1.00$).

Discussion

Over the past two decades, substantial progress has been made in improving the performance of serological tests for the diagnosis of CE and AE (Zhang and McManus 2006; Carmena et al. 2006, 2007; Zhang et al. 2012). Novel test systems were developed and new antigens identified which led to an increase in both diagnostic sensitivity and specificity (Zhang and McManus 2006; Carmena et al. 2006, 2007; Zhang et al. 2012). In addition, several serological tests for the differential diagnosis of CE and AE have been developed (Gottstein et al. 1983, 1986; Ito et al. 1999; Liance et al. 2000; Feng et al. 2010).

Table 1 Number of positive serum samples from patients with cystic echinococcosis (CE) and alveolar echinococcosis (AE) from patients with other diseases and from healthy individuals tested by ELISA and ICT

		No. of positive samples			
		ELISA		ICT	
Serum samples	No. of sera	HCF	rEM18	HCF	rEM18
CE (liver/lung)	144 (137/7)	133 (129/4)	4	131 (127/4)	2
CL	1	1	0	1	0
CE1	15	12	1	12	0
CE2	54	51	1	51	1
CE3	67	65	2	64	1
CE4/CE5[a]	7	4	0	3	0
AE	51	41	50	36	50
AE1	2	0	1	0	1
AE2	8	5	8	3	8
AE3	16	15	16	14	16
AEf	25	21	25	19	25
Hepatic cysts	13	0	0	0	0
Cysticercosis	25	8	0	5	0
Schistosomiasis	15	1	0	0	0
Toxoplasmosis	10	0	0	0	0
Paragonimiasis	8	0	0	0	0
Clonorchiasis	9	0	0	0	0
Healthy donors	60	2	1	1	0

[a] Patients with CE4 and CE5 cysts were grouped together.

In the present study, we devised an ICT for the differential detection of specific antibodies to *E. granulosus* and *E. multilocularis* infections. As antigens, we employed crude sheep HCF (specific for *E. granulosus* but less specific for *E. multilocularis*) and rEm18 (highly specific for *E. multilocularis*). Sensitivity and specificity of our ICT method was similar to a benchmark ELISA employing the same antigens. With respect to diagnosing CE, the sensitivity and specificity of our test were 91 and 97 %, respectively, which compared with other serological tests for CE are rather high (Carmena et al. 2006; Zhang et al. 2012). The actual sensitivity of our ICT may be overestimated due to the composition of the serum collection. It included only few CE serum samples from patients with cysts in the lung (5 %) while the incidence is between 17 and 47 % (Raether and Hänel 2003; Eckert and Deplazes 2004; Mandal and Mandal 2012; Siracusano et al. 2012). However, a large proportion of patients with pulmonary cysts tend to be seronegative (see below). In addition, the serum collection contained a high percentage of serum samples from CE patients with active cysts that usually are seropositive. Therefore, our serum collection may yield higher sensitivity values than expected. The sensitivity and specificity of our ICT for detecting AE were 98 and 99 %, respectively, and thus better than most other serological tests for AE (Carmena et al. 2007). Importantly, our ICT discriminated very well between *E. granulosus* and *E. multilocularis* infections: only 2 (1.4 %) out of the 144 CE patients were found positive for the presence of antibodies against rEm18. Thus, in the present study, we achieved a significant improvement in the differential diagnosis of CE and AE as discrimination between *E. granulosus* and *E. multilocularis* infections was always a difficult problem in the serodiagnosis of the two different forms of echinococcosis.

Table 2 Diagnostic performance of ELISA and ICT in the detection of cystic echinococcosis (CE) and alveolar echinococcosis (AE)

	CE		AE	
Diagnostic parameter	ELISA	ICT	ELISA	ICT
True positive (tp)	133	131	50	50
True negative (tn)	180	185	279	282
False positive (fp)	11	6	5	2
False negative (fn)	11	13	1	1
Sensitivity[a] (%)	92.4	91.0	98.0	98.0
Specificity[b] (%)	94.2	96.9	98.2	99.3
Diagnostic efficiency[c] (%)	93.4	94.3	98.2	99.1

[a] Sensitivity=tp×100/(tp+fn)

[b] Specificity=tn×100/(tn+fp)

[c] Diagnostic efficiency=(tn+tp)×100/(tp+fp+tn+fn)

Although a number of native and recombinant antigens for the immunodetection of *E. granulosus* have been identified and evaluated, most of these antigens do not perform better in serological tests than crude HCF (Zhang et al. 2012). For example, Li et al. (2010) evaluated the IgG antibody response

to recombinant antigen B (rAgB) with serum samples from CE patients from Tibetan communities of northwest Sichuan, China: only 77.6 % (191/246) showed a positive reaction. A higher sensitivity of 91.7 % (33/36) was recently reported by Kalantari et al. (2010) for rAgB when tested with serum samples from Iranian CE patients. In another study, carried out by Tawfeek et al. (2011), only 87.5 % (35/40) of Egyptian CE patients showed a positive IgG antibody response against native antigen B (AgB). In previous work, we compared the diagnostic performance of HCF, native AgB, rAgB/1, rAgB/2, and rAgB/3. With CE serum samples, the five antigens showed positive responses of 90.8, 87.4, 67.8, 78.2, and 59.8 % with specificities of 96.0, 96.0, 96.0, 98.0, and 97.0 %, respectively (Gao et al. 2012b). In the present investigation, we used the HCF antigen for the detection of specific antibodies in CE serum samples because of the high performance of the HCF antigen compared to other antigens despite some drawbacks such as different batch quality and natural variations of this antigen.

In this study, 13 (9 %) out of 144 serum samples from confirmed CE patients did not show a positive reaction. The reason for this may be attributed to low antibody levels in the serum of these CE patients. It is known that the degree of antibody response depends on the location and condition of the mature hydatid cyst in CE patients (Carmena et al. 2007). For instance, hydatid cysts in organs other than the liver are usually associated with lower serum antibody titres (Zhang et al. 2012). This is supported by our findings that only four of the seven serum samples from pulmonary CE patients showed a positive reaction. Furthermore, in about 20 and 40 % of patients with hepatic and pulmonary hydatid cysts, respectively, specific antibodies may not be detectable with certain immunological test systems (Pawłowski et al. 2001). In addition, there is evidence that *E. granulosus* antigens may regulate and inhibit the host antiparasite immune response (Siracusano et al. 2012). Our findings also seem to confirm previous observations that patients with inactive parasites (CE4/CE5) exhibit lower reactivity. The relative low specificity of the ICT is due to cross-reactivity of the crude HCF antigen with serum samples of patients infested with other taeniid tapeworms, in particular with the larval stage of *T. solium*: 5 (20 %) out of 25 serum samples of patients with cysticercosis showed a positive reaction. This is a commonly observed problem in the diagnosis of CE using crude HCF as antigen (Carmena et al. 2006). Part of this cross-reactivity is due to a wide range of antigenic similarities between *Echinococcus* and *Taenia* species (Abuseir et al. 2013). This problem of cross-reactivity may be improved but not overcome by using the recombinant antigen B8/1 (RAg8) instead of crude HCF. Although Mohammadzadeh et al. (2012) demonstrated very recently that serum samples of 20 taeniasis patients showed no cross-reactivity with RAg8, Hernández-González et al. (2012) and our own research (Jun-yun Wang et al., unpublished data) indicated that recombinant antigens of the B family still exhibits cross-reactivity with some serum samples of patients infested with *T. solium*. For this reason, we used the established HCF as antigen for the diagnosis of CE.

In contrast to the HCF antigen, the rEm18 antigen performed much better. Compared with a previously developed ICT using this antigen (Sako et al. 2009), sensitivity and specificity of our ICT were very similar. The present study also confirmed that rEm18 is as good as, or even better than, most other antigens so far evaluated for immunodetection of *E. multilocularis* (Carmena et al. 2007). In addition, our results corroborate previous findings that rEm18 detects almost 100 % of AE cases (Ito et al. 2002; Xiao et al. 2003).

In conclusion, we have developed a simple and rapid ICT for differential diagnosis of CE and AE. The advantage of our ICT is that no expertise, experience, and special equipment is required to perform the test, that only 15 min are needed for evaluation of the test, and that the test is cheaper than other serological tests like ELISA or immunoblot analysis. These advantages make our ICT an ideal primary screening tool for the differential diagnosis of CE and AE in clinical practice and probably in mass screening programs in endemic areas.

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