EVALUATION OF PARTIALLY PURIFIED SOLUBLE EGG ANTIGENS IN COLLOIDAL GOLD IMMUNOCHROMATOGRAPHY ASSAY CARD FOR RAPID DETECTION OF ANTI-*SCHISTOSOMA JAPONICUM* ANTIBODIES

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Abstract. Shistosomiasis is one of the important parasitic diseases in developing countries and especially remains a threat to public health in China. Many immunodiagnostic kits have shown cross-reactions with other parasitic diseases and need large volumes of serum for the tests. In this study, we evaluated partially purified soluble egg antigen (SEA) in a colloidal gold immunochromatography assay (GICA) card kit for rapid detection of anti-*Schistosoma japonicum* antibodies using 5 μl of serum. Partially purified SEA from *S. japonica* was purified by Sephacryl S-300 chromatography. The optional reaction system and detection level of GICA using partially purified SEA were established by improving conjugated concentration and formulation of sample buffer and labeled solution. GICA showed 93.7% sensitivity in detecting schistosomiasis patients, 97.6% specificity in healthy population and patients with other parasitic diseases and a Youden's index value of 0.91. Cross-reaction with other parasitic diseases, such as paragonimiasis (1 case) and toxoplasmosis (1 case) is significantly lower compared to using crude SEA .Partially purified SEA in GICA is practical for detection of schistosomiasis in the field as it requires a small volume of serum, has high sensitivity, and has low cross-reaction rate.

Keywords: *Schistosoma japonicum*, gold immunochromatography assay, soluble egg antigen

INTRODUCTION

Schistosomiasis japonica is one of the important parasitic diseases in developing countries. It is endemic in China, the Philippines, Indonesia and Japan. Among the four endemic countries, schistosomiasis in China causes the heaviest damage to people's health (Chen, 2012). By the end of 2010, in China a total of 325, 824 cases of schistosomiasis japonica were estimated and 453 counties were endemic areas where the population was about 0.244 billion .A total of l,476,606 cattle were raised in schistosomiasis transmission regions and the infection rate of cattle infected with *S. japonicum* was 1.04% (Lei *et al*,

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2011). Most schistosome-endemic areas in China are characterized by low-intensity infection that is independent of prevalence (Yu *et al*, 2012). Thus, schistosomiasis japonica remains a threat to public health in China. Schistosomiasis is also one of the neglected tropical diseases in the developing world (Utzinger *et al*, 2010).

It is certainly true that prompt diagnosis and surveillance of schistosomiasis are needed for treatment and control of the disease. Immunodiagnostic approach is usually employed as epidemiological tools for screening of targeted populations in schistosome-endemic areas and as auxiliary diagnostic tools for individual case (Wu, 2002). However, many immunodiagnostic kits currently using crude soluble egg antigen (SEA) are based on detection of schistosome-specific antibodies (Xin *et al*, 2006), and have been shown to cross-react with other parasitic diseases (Zhu *et al*, 2012). The reason is that SEA component is complex with a number of cross-reacting antigens to other parasitic species, thereby restricting the efficiency of the diagnosis (Huang and Zhuge, 2008).

The advantages of gold immunochromatography assay (GICA) include simple operation, rapid reaction, and no need of any special equipment and staff training. Not only can it be used for epidemiological investigations, as immunodiagnostic tool but also can serve in detection of an individual case. WHO has praised it as a 'high technology production method and low technology application' (WHO, 1996). However, compared with ELISA, sensitivity in the immunodiagnosis of schistosomiasis by GICA is lower and the serum volume required is larger (Lin *et al*, 2010). These disadvantages have resulted in its restricted use in field applications. Therefore, the purpose of this study was to develop and evaluate a new GICA test using partially purified *S. japonicum* SEA. This developed GICA test was also improved from the original GICA for schistosomiasis detection and applied to a field study.

MATERIALS AND METHODS

SEA preparation

Crude **S**EA was prepared as described by Jiang *et al* (2007), which employs Sephacryl S-300 chromatography (BioLogic LP chromatography **s**ystem; BioRad, Hercules, CA) combined with ultrafiltration. The column was equilibrated with elution buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2), then 2.4 mg of SEA in 2 ml of elution buffer were applied to the column and eluted at a flow rate of 1 ml/min. The protein fractions with molecular weights > 10 kDa were collected, pooled and then centrifuged in Amicon Ultra PL-10 (10kDa cut-off) ultrafiltration unit (Millipore, Billerica, MA) at 5,000*g* for 20 minutes. Then, 0.01 M phosphate buffer, pH 7.2 was added and the centrifugation was repeated to yield the partially purified SEA .

Preparation of GICA card teat kit

Colloidal gold particles, 35 nm diameter, were prepared by the sodium citrate reduction method (Frens, 1973). An aliquot of 495 ml of distilled H_2O containing 5 ml of 1% chloroauric acid was heated to 100ºC, then 5 ml aliquot of 1% trisodium citrate was added and the solution was boiled for 10 minutes when the color of the solution changed from dark red to purple. After adjusting pH to 8.0, 7.5 mg of partially purified SEA were added, and after 30 minutes 100 ml aliquot of 5%bovine serum albumin (BSA) was added and the solution was centrifuged at 10,400*g* for 30 minutes. The pellet was dispersed in an appropriate volume of preservation solu-

tion (0.01 M phosphate buffer, pH 8.0, 1% BSA) to produce the colloidal gold-labeled solution. A 0.8 cm x 30 cm polyester film (6613 type; Ahlstrom, Helsinki, Finland) was immersed in the solution for10 minutes, baked at 42ºC for 6 hours.

The test band (T band) consisted of varying concentrations of partially purified SEA (or crude SEA as comparison) dissolved in coating solution (0.01 M phosphate buffer, pH 7.2), and the control band (C band) consisted of varying concentrations of rabbit anti-SEA antibodies in coating solution (Shen and Zhou, 2002). The bands were dispensed using non-contact BioJet (model XYZ3050, BioDot, Irvine, CA) at 1 µl/cm on a 2.2 cm wide nitrocellulose membrane film (M135 type, Millipore, Billerica, MA) attached onto a polyvinyl chloride (PVC) pad, which was then placed on top of the baked polyester film coated with colloidal gold preparation. This was cut into 3 mm width strips using an automated cutting machine (model CM-400, BioDot, Irvine, CA), placed in a plastic card box, which was in turn put into an aluminum foil bag and stored at the room temperature.

To conduct the GICA card test, a 5 µl serum aliquot was added to the sample hole, followed by 1 drop of sample buffer solution. The results can be obtained within 10- 20 minutes. T band and C band should appeared together if the result is positive, and only the C band if the result is negative.

Serum samples

From the schistosomiasis endemic area of Jiangxi Province, China sera were collected from 174 patients with schistosomiasis japonica infection, comprising 103 cases with < 25 eggs/g of stool and 71 cases with > 25 eggs/g of stool as determined using Kato-Katz technique; 170 control sera were collected from healthy non-infected individuals, determined by stool examination, living in non endemic areas of Shanghai. Sera from subjects with other parasitic diseases included 18 cases with paragonimiasis, 20 with clonorchiasis, 10 with cysticercosis, 10 with trichinellosis and 10 with cystic echinococcosis, obtained from the National Institute of Parasitic Disease, China CDC. In addition, 15 sera with falciparum malaria or vivax malaria, determined by microscopic examination, were collected from an malaria endemic area in Yunnan Province; 10 sera with entamoebiasis histolytica, determined by stool examination of iodine smears, received from the Central Hospital of Shanghai Changning District; and 17 sera with toxoplasmosis, obtained from the Danish National Research Institute for Serology, Copenhagen. For field study, 507 sera for surveillance of schistosomiasis were collected from a population working in Shanghai but came from three schistosomiasis endemic provinces of Anhui, Hunan and Hubei. Antibodies against *S. japonicum* from this cohort were detected in parallel by GICA and ELISA (Shenzhen Reagent, Guangdong, China).

"National reference for schistosomiasis antibody diagnostic reagents" (Lot: 20041108) were provided by the State Verification Institute of Pharmaceutical and Biological Products. Negative reference samples consisted of 15 healthy control sera, and positive reference samples consisted of 15 sera positive to antibodies of schistosomiasis. The lowest positive reference serum was diluted from 1:4 to 1:32 for the minimum detection limit for GICA card test kit. For precision of GICA card test kit, positive reference sera were assayed 10 times. For stability test of the GICA, the kit was kept at 37ºC for 60 days or at room temperature for 15 months and

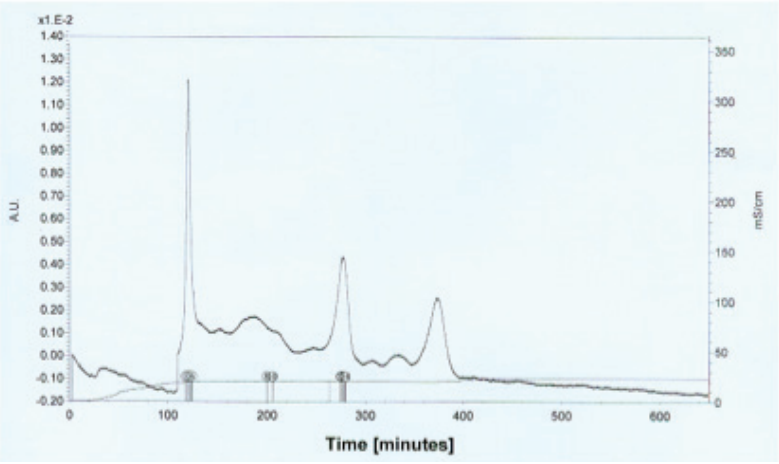

Fig 1– Sephacryl S-300 column chromatography elution profile of *S. japonicum* SEA. The column was equilibrated with elution buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH7.2), then 2.4 mg of SEA in 2 ml of elution buffer were applied to the column and eluted at a flow rate of 1 ml/min.

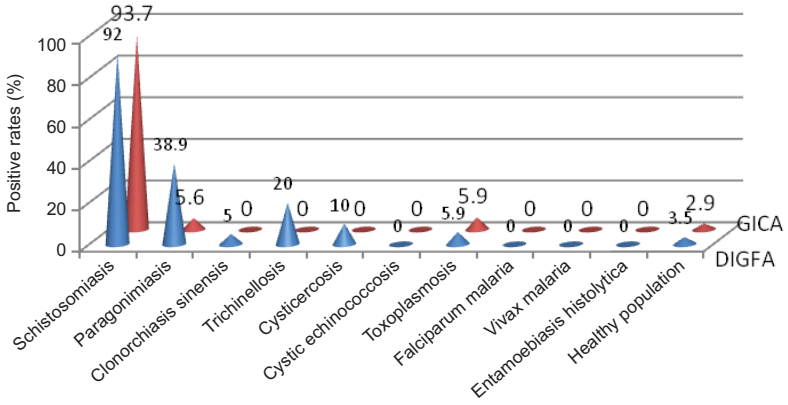

Fig 2 - Positive detection rates of antibodies to schistosome in patients with parasitic diseases and healthy control population with DIGFA using crude *S. japonicum* SEA and GICA using partially purified SEA.

then used in the detection of 15 negative and 15 positive reference samples.

Statistical analysis

SPSS 16.0 was used to evaluate chi-square, Kappa value and consistency of the results from GICA card test kit, DIGFA kit and ELISA method.

RESULTS

SEA separated by Sephacryl S-300HR column chromatography into 3 protein peaks (Fig 1) and protein fractions of the first and second peak (> 10 kDa) were collected. The protein concentration of the 3 peaks was 1.05 mg/ml, 0.55 mg/ml and 0.17 mg/ml, respectively. Using the partially purified antigen from pooled peaks 1 and 2, sensitivity was 93.7% and specificity for healthy population and patients with other parasitic diseases was 97.6%. Cross-reactivity with other parasitic diseases is significantly lower using the partially purified compared to crude SEA (1.6% and 8.8%, respectively ($\chi^2 = 6.57$, $p < 0.01$) (Table1).

Under optimal conditions, coating concentration of T band was 0.8 mg/ml and that of C band 5 mg/ml, labeled colloidal gold buffer was 0.01 M PBS pH 8.0 containing 1% BSA, polyethylene glycol and sucrose; and sample buffer was 0.02 M PBS pH 8.0 containing 1% BSA and polyethylene glycol. Under these conditions, the GICA card kit using partially purified SEA, consistently produced positive

Table1
Comparison of antibody detection of schistosomiasis by GICA card using crude and partially purified S. *japonicum* SEA.

Group	No. of cases	Crude SEA		Partially purified SEA	
		No. of positive cases	Positive rate (%)	No. of positive cases	Positive rate (%)
Schistosomiasis	174	165	94.8	163	93.7
Paragonimiasis	18	6	33.3	1	5.6
Clonorchiasis sinensis	20	2	10.0	0	0
Trichinellosis	10	2	20.0	0	0
Cysticercosis	10	0	0	0	0
Cystic echinococcosis	10	0	0	0	0
Toxoplasmosis	17	1	5.9	1	5.9
Falciparum malaria	15	0	0	0	0
Vivax malaria	15	0	0	0	0
Entamoebiasis histolytica	10	0	0	0	0
Healthy population	170	7	4.1	5	2.9

results for 5 µl of 1:32 diluted minimum positive reference sample as the results using 10 µl or 20 µl of the same diluted reference sample. Employing the national reference for schistosomiasis antibodies diagnostic reagents, both the 15 negative and 15 positive reference test samples produced coincidence rate of 100%; 10 tests of precision were all positive; the minimum detection limit was 1/32 of the national reference sample; and was 8 times more sensitive than the to dipstick method. The GICA card stored at 37°C for 60 days or at room temperature for 15 months, tested with 15 negative and 15 positive reference samples, produced 100% coincidence rate in both cases.

Comparing the developed GICA card kit with the dot immunogold filtration assay kit (DIGFA, Shanghai Shejie, China) that is also based on labeled colloidal gold technique, there is no significant difference between the two methods as regards sensitivity of detecting anti-schistosoma antibodies from schistosomiasis patients (93.7% and 92%, respectively) and specificity for healthy population and patients with other parasitic diseases (97.6% and 93.9%, respectively). The Youden's index value with both methods was 0.91 and 0.86, respectively. Cross-reaction rate of GICA card kit with paragonimiasis and toxoplasmosis was 5.6% and 5.9%, respectively, but no cross-reaction with other parasitic diseases was apparent (Table1, Fig 2). Total cross-reaction rate with other parasitic diseases using GICA card kit was 1.6%, much lower than with DIGFA (9.6%) (χ^2 =7.57, p <0.01).

Surveillance of schistosomiasis in migrant population in Shanghai City using both GICA card and ELISA kit showed 35 positive and 457 negative cases from a total of 507 cases by both methods, but different results in 15 cases. Total coincidence rate reached 97.0% (492/507), with Kappa value of 0.81, showing both tests have good consistency.

DISCUSSION

S. japonicum SEA protein antigens recognized by immune sera are mainly > 38 kDa (Zhou *et al*, 2000) and proteins of 270, 151, 78, 73, 70, 69, 50 and 24 kDa are recognized during the early stages of infection (Hua *et al*, 2005; Wang *et al*, 2012). The 31-32 kDa protein fraction from viable SEA is highly immunogenic, having the least cross-reaction with other parasitic infections, and may be a useful serologic marker for diagnosing and differentiating between early and chronic *S. mansoni* infection (Hussein *et al*, 2004). These results all suggest potential field applications of these purified immunodominant SEA proteins in the establishment of rapid diagnostic kits.

Column chromatography is a rapid, simple and reliable method for isolation and purification of antigens of *S. japonicum*. Proteins of 26~28 kDa from immature SEA isolated through super gel column chromatography (ACA54) retain their biological activity and function (Wang *et al*, 1996). Eleven distinct fractions from SEA separated by DE22 cellulose chromatography included 9 immunoreactive fractions that contained the main immune components of crude SEA and eliminated the major proteins irrelevant to the immunogenicity of *S. japonicum* (Jiang *et al*, 2007).

In this report we separated SEA antigens using Sephacryl S-300HR column chromatography and pooled protein fractions of > 10 kDa showed very good antigenicity and high specificity to schistosoma antibodies. On this basis and with further improvement to the SEA-labeled colloidal gold formulation, we established a GICA card that not only met all requirements of the national reference standards set up by the National Standardization Committee for quality control of schistosomiasis antibody diagnostic kits, but also exceeded the lowest detection value of the national reference by 8 folds. There is significant difference between GICA card and DIGFA kits regarding cross-reaction rate with other parasitic diseases. This may have been due to the use of crude SEA using by DIGFA. Field application of GICA for surveillance of schistosomiasis in the migrant populations of Shanghai City coming from schistosomiasis endemic regions showed a high consistency with the results of ELISA method that had been selected as the disease detection reagent in the Third National Schistosomiasis Epidemiological Sampling Survey (Xu *et al*, 2005), and very similar to the recent surveillance results in Shanghai (Zhou *et al*, 2007). The amount of serum used for detection by GICA card was 5 μl, and the diagnostic performance was not reduced, especially for positive detection rate of the low degree infection sera, which will be extremely valuable in diagnosis and surveillance of regions with low endemicity for schistosomiasis. Furthermore, the 5 μl of serum is the minimum volume for schistosomiasis detection using dipstick kit at present, so making it convenient for staff sampling on-site. They only need to collect 10~15 μl of blood from a finger prick and so reduce the subjects of pain and psychological trauma, making the procedure more likely to be accepted and facilitates cooperation with the inspection.

As the antigen labeled in colloidal gold is partially purified SEA, the developed GICA card can be used directly to detect anti-SEA antibodies from humans and animals. Cross-reaction rate with other parasitic diseases using the partially purified SEA was lower than that using crude SEA, but whether the cause is related to small molecular fractions of

SEA needs further research. In conclusion, GICA card kit using partially purified *S. japonicum* SEA has advantages, including low amount of serum needed, lower cross-reaction and wider applications that makes the procedure more suitable for epidemiological surveys, disease screening and immunological diagnosis in low endemic areas of schistosomiasis.

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