Evaluation of an IgY-Based Immunomagnetic Enzyme-Linked Immunosorbent Assay System for Detection of Circulating *Schistosoma japonicum* Antigen in Serum Samples from Patients in China

Jia-hui Lei, Bing-tao Su, Hong Xu, Ji-long Shen, Xiao-hong Guan, Zhen-qing Feng, Yong-long Li, Ming-xing Xu, and Wen-qi Liu*

Department of Parasitology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People's Republic of China; Department of Parasitology, Anhui Medical University, Hefei, People's Republic of China; Key Laboratory of Antibody Technology of the Ministry of Health, Nanjing Medical University, Nanjing, People's Republic of China; Institute of Schistosomiasis Control, Wuhan Center of Disease Control, Wuhan, People's Republic of China

Abstract. We have developed a novel egg yolk antibody (IgY)-coated magnetic beads antigen-capture immunoassay for detection of a circulating antigen of *Schistosoma japonicum* in serum samples of patients in schistosomiasis-endemic areas of China. This IgY-based immunomagnetic bead enzyme-linked immunosorbent assay (IgY-IMB-ELISA) uses polyclonal IgY-coated magnetic beads as a capture antibody, and a monoclonal IgG as a detection antibody. The sensitivity of the magnetic immunoassay was 100% (40 of 40) in cases of acute infection and 91.5% (107 of 117) in chronic cases of schistosomiasis, and no positive reaction was found in 0 of 49 healthy persons. Cross-reactivity was 3.3% (1 of 33) with clonorchiasis and 0% (0 of 20) with paragonimiasis. There was a significant correlation between ELISA absorbance value and egg count (eggs per gram feces) and a correlation coefficient of 0.88 in a small sample of 14 patients. The results demonstrated that the IgY-IMB-ELISA is a sensitive and specific assay for detection of human schistosomiasis japonica.

INTRODUCTION

Schistosomiasis is a serious tropical disease caused by worms of the genus *Schistosoma*, which infect humans and most livestock throughout the world. This parasitic disease ranks second to malaria and affects an estimated 200 million persons in developing countries in tropical and subtropical regions.^{1,2} Ambitious goals and strategies have been set for the control of this infectious disease by governments and many organizations in collaboration with the World Health Organization.³ For the evaluation and monitoring of the epidemiologic situation, especially in areas where prevalence and intensity of infection have been brought to low levels through control, the more progress control programs make, the more crucial the need becomes for an accurate diagnostic technique.⁴

In China, determination of target populations for chemotherapy in schistosomiasis-endemic areas and assessment of control activities are built on the outcome of diagnostic tests, and diagnosis of schistosome infection depends on parasitologic or serologic techniques.⁵ Fecal smear or miracidial hatching and the indirect hemagglutination assay (IHA) have been the two most widely field-used approaches,⁶ although the poor sensitivity of fecal egg detection strongly underestimates the prevalence,^{7,8} and antibody detection serologic tests fail to differentiate present and past infections.⁴ Development of better diagnostic protocols based on antigen detection with increased sensitivity and specificity is central to effective surveillance programs.^{6,9}

Several immunologic tests have been described to detect schistosome circulating antigens in diagnosis of *Schistosoma* infection. These tests include IHA, immunofluorometric assay, sandwich enzyme-linked immunosorbent assay (ELISA), magnetic bead immunoassay,⁶ hybridoma cell agglutination,¹⁰ and an antigen-detection strip test.¹¹ These tests exhibited var-

ious sensitivities and specificities and rely on antibodies used and intensity of infection.

The yolks of immunized chickens are an abundant and economical source of polyclonal antibodies. Specific egg yolk antibody (IgY) offers several considerable advantages over mammalian antibodies.¹² Because of the phylogenetic distance between birds and mammals, chicken antibodies recognize more epitopes when mammalian proteins are used as antigens than the corresponding mammalian antibodies. Because chicken IgY does not cross-react with mammalian IgG and does not bind bacterial components or mammalian Fc receptors,¹² non-specific binding is reduced, and the need for cross-species immunoabsorptions is also decreased. Therefore, chicken IgY has significant advantages over IgG as the first antibody in some types of immunologic assays.

An immunomagnetic bead-based immunoassay is a popular approach in diagnosis of many food-borne and infectious diseases. This innovative technique involves immobilizing antibodies on micro-sized paramagnetic beads and uses antibody-coated beads to trap antigens from liquid media. Furthermore, the small size and shape of the micro-beads enables them to be evenly dispersed in the sample for improving the effectiveness of the antibody conjugation, and consequently enhance the sensitivity of antigen detection.^{13,14}

Recently, a novel IgY-based immunomagnetic bead sandwich ELISA (IgY-IMB-ELISA) was established in our laboratory to detect circulating antigens in serum samples from mice with murine schistosomiasis japonica.¹⁵ In this previous study, we produced polyclonal IgY from chickens immunized with *S. japonicum* soluble egg antigen (SEA), which showed a high specificity and a high concentration of detection (average = 69 mg per egg). The high-quality IgY was then coupled to commercial magnetic beads and used as a capture antibody in sandwich ELISA. The circulating antigen in serum samples of mice with schistosomiasis japonica could be detected by IgY-IMB-ELISA as early as four and five weeks after infection. Moreover, this assay was valuable in the assessment of praziquantel treatment for mice with schistosomiasis.

This study reports analysis of this IgY-IMB-ELISA for detection of circulating *S. japonicum* antigen in serum samples

* Address correspondence to Wen-qi Liu, Department of Parasitology, Tongji Medical College, Huazhong University of Science and Technology, No. 13 Hangkong Road, Wuhan 430030, People's Republic of China. E-mail: liu_wq2002cn@yahoo.com.cn

of patients living in schistosomiasis-endemic areas in China. The results have been also compared with those from a typical IHA, and the association with fecal egg output was examined.

MATERIALS AND METHODS

Human serum samples. A total of 536 serum samples were collected for the present investigation. We tested 157 schistosomiasis cases from three schistosomiasis-endemic villages for schistosomiasis japonica in Hubei Province and Anhui Province, China. These cases were confirmed as parasitologically positive by using the Kato-Katz method with three fecal samples or by miracidial hatching assay. Of these cases, 40 had been defined as acute according to exposure history and clinical manifestation; the others had been defined as chronic schistosomiasis cases. Egg counts of 14 patients from national surveillance of schistosomiasis japonica were used in a comparative analysis with ELISA absorbance. An additional 277 serum samples collected from the same schistosomiasis-endemic areas showed negative results in fecal tests, of which 248 showed positive results in the SEA-IHA. Serum samples were obtained from a population of 49 healthy persons living in Shandong Province (non-endemic for schistosomiasis) and used as controls.

Two groups of 53 patients with either clonorchiasis (33 patients) or paragonimiasis (20 patients) living in Anhui Province were also used to assess cross-reactivity. Patients with clonorchiasis sinensis were confirmed by clinical examination and detection of eggs in feces. The cases infected with *Paragonimus westermani* were diagnosed by exposure history, clinical manifestations, and a serologic test. Co-infection with *S. japonicum* was excluded in both groups on the basis of exposure history, egg examination result, or serologic test result.

All serum samples were stored at -20°C until use. All experimental work conformed with local government regulations that in turn complied with Chinese national laws on human ethics. Informed consent was obtained from all adult participants or from parents of minors.

SEA-IHA detection. An SEA-IHA was used to detect the antibody against schistosome SEA in all serum samples collected, although the results of the same IHA have been reported for some cases. The IHA was performed as described by Zhou and others,⁵ and the IHA kit was kindly provided by the Hubei Center of Disease Control and Prevention. The reaction was conducted in V-shaped microtiter plates (Greiner, Frickenhausen, Germany). The IHA titer was obtained by using the dilution of a serum sample tested. If a titer was ≥ 10 , the test results was considered positive.¹⁶

Antibodies against *S. japonicum* and conjugation. Preparation and characterization of chicken anti-SEA polyclonal IgY has been described.¹⁵ Carboxyl-activated magnetic beads (10% [v/v] suspended in 0.01 M phosphate-buffered saline [PBS], pH 7.0, diameter = 500 nm) were obtained from the Mag-Century Biotechnology Company (Beijing, China). Coupling with IgY was performed as described.^{13,15} Briefly, 2.5 mL of magnetic bead suspension was coated with 450 μg of polyclonal IgY and resuspended in 0.05 M PBS, pH 7.6, containing 0.1% bovine serum albumin. The bead-bound IgY was used as an antigen-capture antibody in the ELISA.

A monoclonal IgG NP28-5b against SEA was generated and maintained in the Key Laboratory of Antibody Technology of the Ministry of Health, Nanjing Medical University (Nanjing,

China). NP28-5b was purified from supernatant of a cell culture by using affinity chromatography (Bio-Rad Laboratories Ltd., Shanghai, China). This antibody recognized a band with molecular mass of 140,000 D in SEA when analyzed by Western blotting. Conjugation of NP28-5b to horseradish peroxidase (HRP) was performed by using the NaIO_4 oxidation method. The HRP-labeled NP28-5b was used as a detection antibody at a 1:1,000 dilution in the ELISA.

IgY-IMB-ELISA. All reactions were conducted at room temperature, and each incubation was conducted at 37°C . A total of 50 μL of IMB (10 μg of IgY) and 100 μL of serum sample at a 1:2 dilution in 0.01 M PBS, pH 7.4, were placed in a flat-bottom reaction tube, which was fitted to a Magnetic Particle Rack (Bioekon Inc., Beijing, China). The mixture was rocked sufficiently to prevent settling of the IMB for one hour on a Speci-Mix VXR56 (Bioekon Inc.). Immunomagnetic separation of the beads was performed by placing the tubes on the rack of the magnetic holder for two minutes, and the supernatant was removed and discarded. The IMB were resuspended by gentle vortexing with 250 μL of PBS containing 0.5% Tween-20, and samples were washed three times. A total of 100 μL of HRP-conjugated NP28 5b (at a 1:1,000 dilution) was added to the IMB, mixed for one hour, and separated by placing the mixture on the rack of the magnetic holder for two minutes. The liquid was removed as described above. After a final washing (six times) in 250 μL of PBS containing 0.5% Tween-20, 100 μL of substrate (tetramethylbenzidine dihydrochloride; Tiangen Biotech Ltd., Beijing, China) was added and incubated for 15 minutes with gentle shaking. The reaction was stopped by adding 30 μL of 2.0 M sulfuric acid, and the IMB were separated by placing the tube on the magnetic holder for three minutes. The supernatant was transferred to the microtiter plate (Greiner, Frickenhausen, Germany) and the optical density (OD) was measured at 450 nm in a ELISA reader (Thermo Labsystems, Vantaa, Finland).

Serum samples from healthy persons and PBS were used as negative controls and a blank control, respectively. All results were recorded after appropriate blank correction. One serum sample was considered positive if the value was at least two times higher than that of the negative control sample. Each sample was tested in duplicate in three separate tests.

Data analysis. The sensitivity of a given test was defined as the percentage of serum samples among fecal test-positive persons (gold standard) showing an OD value two times higher than that of the control. The rate of cross-reactivity was defined as the number of persons who had positive results as a proportion of the total number of persons without schistosomiasis, but with other common helminth infections, which were represented by persons with clonorchiasis and paragonimiasis in this study. Differences between percentages were compared by using the chi-square test as needed. The Student's *t* test was used to compare means, and *P* values < 0.05 were considered significant.

RESULTS

Results for detection of *S. japonicum* infection for all serum samples by SEA-IHA and results of a stool test (Kato-Katz or miracidial hatching) are shown in Table 1. All definitive schistosomiasis cases were positive in this antibody detection, and no positive results were found in a healthy population living in

TABLE 1

Reactivity of serum samples from persons with different parasitic diseases, China, in an IgY-IMB-ELISA for detection of circulating antigen and an SEA-IHA for detection of antibodies*

Parasitic status	No. serum samples	Positive by IgY-IMB-ELISA		Positive by SEA-IHA	
		No.	%	No.	%
Acute schistosomiasis	40	40	100.0	40	100.0
Chronic schistosomiasis	117	107	91.5	117	100.0
Egg negative, IHA positive	248	14	5.6	248	100.0
Egg negative, IHA negative	29	2	6.9	0	0
Clonorchiasis	33	1	3.0	4	12.1
Paragonimiasis	20	0	0	3	15.0
None	49	0	0	0	0

* IgY-IMB-ELISA = IgY-based immunomagnetic bead enzyme-linked immunosorbent assay; SEA = soluble egg antigen of *Schistosoma japonicum*; IHA = indirect hemagglutination assay. Each sample was assayed in duplicate tubes in three experiments.

a non-endemic area. In the population living in schistosomiasis-endemic areas but with a negative schistosome egg detection result, our IHA results also showed that 248 persons were antibody positive, which was consistent with previous antibody detection results obtained from local Schistosomiasis Control Station or schistosomiasis-specific clinic. Four and three positive results were reported from groups with clonorchiasis and paragonimiasis, respectively.

We developed the IgY-IMB-ELISA to detect circulating antigen in serum samples of persons with schistosomiasis. Results for 605 persons from three schistosomiasis-endemic areas of China by IgY-IMB-ELISA and results of our SEA-IHA are shown in Table 1. We used the results of the Kato-Katz test or miracidia hatching as the gold standard of diagnosis, which showed a sensitivity for IgY-IMB-ELISA of 100% (40 of 40) in cases of acute infection and 91.5% (107 of 117) in chronic schistosomiasis cases. The overall sensitivity of the magnetic ELISA in circulating antigen detection was 93.6%. The mean $\pm$ OD value for acute cases (2.50 ± 0.39) was higher than that for chronic cases (2.24 ± 0.51), but the difference was not statistically significant. A correlation between OD values for the IgY-IMB-ELISA and fecal egg output (recorded as eggs per gram of feces [EPG]) for 14 persons is shown in Figure 1. These results were similar to the results of Kato-Katz smears from the Hannan Schistosomiasis Control Station of Hubei Province. In this group, the EPG ranged from 2 to 485, but a correlation was found between the OD value and number of eggs excreted ($R^2 = 0.88$). There was no positive reactivity in healthy persons. The rate of cross-reactivity

was 3% (1 of 33) for persons with clonorchiasis and 0% for persons with paragonimiasis.

Egg-negative persons showed an interesting IgY-IMB-ELISA result. Fourteen serum samples showed positive reactions in the group with IHA-positive results, and two persons showed positive reactions even when IHA results were negative. However, the OD values for these two egg-negative persons were low. The overall mean OD value of these egg-negative persons was 1.95. Thus, the IgY-IMB-ELISA showed a positivity rate of 5.8% (16 of 277) in egg-negative persons, including persons who had positive and negative results in the IHA. There was insufficient data to define these positive reactions as false positive because of the low sensitivity of the fecal test, particularly in the well-controlled areas.

DISCUSSION

The achievements of schistosomiasis control program in China over the past 20 years are well known. Through a combination of praziquantel-based chemotherapy and molluscicides, prevalence and intensity of infection have dramatically decreased to an average of 2.5%.¹⁷ Definitive and accurate diagnosis is increasingly required for monitoring locality prevalence and severity of the disease. Presently, selective chemotherapy with praziquantel is being widely used, including by national schistosomiasis programs.^{5,7,18} Identification of populations to be targeted for individual treatment and broad-spectrum chemotherapy in schistosomiasis-endemic areas, assessment of chemotherapy efficacy, morbidity, and evaluation of control strategies need to be based on reliable and available diagnostic tools. Fecal detection lacks sufficient sensitivity and patient compliance.^{19,20} Serologic tests, although they are sometimes well-accepted and show high sensitivity, cannot differentiate between present and past infections in surveillance and thus cannot identify persons for treatment,²¹ and cannot detect frequent reinfection of young laborers in rural schistosomiasis-endemic areas in China.⁵ Since the 1980s, detection of circulating antigens secreted by living parasites has been considered the way to distinguish between active and past infections.^{22–24} Nevertheless, poor sensitivity, such as that in parasitologic methods, limits application in large-scale and individual diagnosis.^{7,8,17}

Some studies have demonstrated that detection of egg antigens provide greater diagnostic sensitivity and specificity than detection of worm antigens for detection of schistosomiasis.^{25,26} Our previous study also indicated that this SEA capture magnetic ELISA is valuable in diagnosis of murine schistosomiasis because of its high sensitivity and specificity, and also has

FIGURE 1. Correlation between optical densities (ODs) obtained in an IgY-based immunomagnetic bead enzyme-linked immunosorbent assay and *Schistosoma japonicum* egg counts (egg per gram of feces) (EPG) in 14 serum samples from persons with chronic schistosomiasis, China. The EPG ranged from 2 to 485, which is represented by their logarithmic transformation along the x-axis. A high correlation was found between the OD values and egg counts ($R^2 = 0.88$).

the potential to be useful in chemotherapy assessment.¹⁵ In the present study, we used magnetic beads conjugated with IgY against SEA as a capture antibody and a monoclonal antibody against SEA as a detection antibody and developed a similar IgY-IMB-ELISA to diagnose *S. japonicum* infection in serum samples of patients. Results showed a sensitivity of 93.6% and a specificity of 100% when parasitologic test results were used as a reference. Cross-reactivity with other trematodiasis was low.

Many immunologic tests based on antigen detection^{27,28} and molecular or proteomic diagnostic techniques^{29,30} have been well studied and described. However, an affordable, easy-to-handle, sensitive and specific method is not yet available.³¹ The sensitivities of antigen-detection immunologic tests were reported as insufficient (range = 60–90%)^{6,10,11} depending on methods used, antigens targeted, and infection intensities of population examined. Well-established molecular techniques (traditional coprologic polymerase chain reaction [PCR] and real-time PCR) were shown by different laboratories to be sensitive for lower-intensity infections. Two independent research groups in Brazil showed sensitivities of 91% and 96%,^{32,33} respectively. A real-time PCR also showed increased sensitivity in a Chinese population.²⁹ Another Chinese laboratory recently showed a higher sensitivity of 96.7% in a less laborious DNA amplification procedure known as loop-mediated isothermal amplification.³⁴ These results provided a useful tool for routine diagnosis in clinical settings. However, the requirement of expensive equipment and professionally trained technicians may impair their application for mass surveillance in rural villages, and additional costs should be weighed.³⁴ Thus, antigen-detection methods with high sensitivity and practicability might be a proper alternative for individual diagnosis and field surveys.

All serum samples were checked primarily by a routine IHA to detect antibody against egg soluble antigen. Results showed 100% antibody positivity in the parasitologically positive defined population and in persons with IHA-positive results. However, an additional 277 persons with stool-negative results were also IHA positive. These infections cannot be differentiated as past infections in persons who were given praziquantel or present infections, but show a low infection intensity that was not detected by the fecal test. Comparatively high cross-reactivity rates of 12.1% and 15% were found in persons with clonorchiasis and paragonimiasis, respectively, which indicated that antibody detection might fail to distinguish other trematode infections from schistosomiasis.³¹

In this study, the magnetic ELISA uses egg yolk polyclonal antibody IgY-coated magnetic beads instead of traditional microtiter plates. The total time required for the test (2.5 hours) may be further reduced by reducing the first washing after serum sample incubation without any impairment of detection. A total of 0.1 mg of beads and 0.01 mg of coated IgY were used per tube, and the lower detection level was 5 ng/mL of SEA with a linear increase from 5 to 80 ng/mL.¹⁵ This finding was not as efficient as the result of Gundersen and others (0.7 ng of circulating anodic antigen detected by a magnetic beads ELISA in an African population.³⁵ However, the present study showed a higher sensitivity (93.6% versus 90%) in a Chinese population.

Our results for the magnetic ELISA also showed a positive correlation between OD and egg excretion (Figure 1). Feldmeier and others³⁶ showed that the amount of circulating

schistosome antigen was closely related to worm burden in a mouse host. Our results support their conclusion by showing a correlation between the light absorbency intensity of the immunoassay and egg output in human disease, despite a small sample of only 14 cases. Moreover, monoclonal antibody NP28-5b against SEA was used in this immunoassay as a detection antibody to facilitate the novel IgY-based magnetic ELISA. Use of the antibody resulted in an assay specificity of 100% and low rate of cross-reactivity. To date, the high degree of cross-reactivity with clonorchiasis and paragonimiasis remains a problem in serologic assays in China because these food-borne trematodiasis are highly endemic and overlap the areas to which schistosomiasis is endemic.³⁷

Because of evolutionary differences for results of the assay, factors contributing to its efficacy include the idea that chicken IgY recognizes more epitopes on mammalian antigens, which results in amplification of the signal.^{38,39} This idea has been confirmed by our previous study in light infections in mice.¹⁵ In addition, immunomagnetic beads of high quality improve the effectiveness of antibody conjugation, thereby enhancing the sensitive capacity of the traditional ELISA.⁴⁰ A modification of magnetic particles is needed to provide high-binding capacity and dispersion stability in magnetic bead-based techniques, especially in diagnostic studies.⁴¹

It is difficult to explain the positive reaction for the magnetic immunoassay found in egg-negative cases with or without a positive reaction in the IHA. Despite the IHA result, because it only represents antibody level, which cannot adequately overcome the presence of an immunologic scar, these positive results should be carefully identified. A positive percentage of 5.8% in a 277 egg-negative persons is acceptable, and greater recognition of polyclonal IgY may improve sensitivity when compared with traditional sandwich ELISA that use mammalian IgG. This higher sensitivity can be verified by a positive reaction in our study resulting from an individual infection with an EPG as low as 2. However, the Kato-Katz method fails to detect eggs when the EPG is < 10; thus, the true prevalence may be underestimated.^{6,7}

Miracidia detection is progressive because more stool is used for hatching eggs, but provides no significant improvement on the sensitivity. It is more likely that the 16 positive persons have true positive results, although positive results were not found in fecal detection tests. Therefore, compared with fecal collection and examination (fecal smear or miracidial hatching), blood collection and established IgY-IMB immunoassays based on antigen detection show advantages because of their improved sensitivity, compliance, and practicability for both patients and technicians, and because no professional training is required for them compared with the PCR. These benefits and improvements indicate that the IgY-IMB assay is an easy-to-handle, economical, specific and stable approach in definitive diagnosis of schistosomiasis, especially in application of field surveillance, as a strong supplement or replacement for laborious parasitologic methods.

In schistosomiasis-endemic areas with low prevalence, false-negative results in parasitologic tests and false-positive results in antibody-based serologic tests are common.^{16,26} However, the relative lack of sensitivity of the antigen detection method could not be excluded in the antibody-positive but egg-negative or antigen-negative cases. This problem will increase as control programs cause infection intensities to decrease even further. Much more attention should be given to improvements in

antigen-detection techniques and proper alliance of antigen-target and antibody-target methods.

Overall, our results show that the IgY-IMB-ELISA has high sensitivity, compliance, and practicability, and can be a potential alternative for field diagnosis of human schistosomiasis. However, more research is needed for schistosomal diagnosis.^{25,29,31,42} Our future studies will focus on cost-effectiveness (time, particle resource, and apparatus), precision, simplicity, and stability of the assay. In addition, evaluation of the IgY-IMB-ELISA by assessment of chemotherapy efficacy in human schistosomiasis is needed.

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Authors' addresses: Jia-hui Lei, Bing-tao Su, Hong Xu, Yong-long Li, and Wen-qi Liu, Department of Parasitology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People's Republic of China, E-mails: lejiahui@hotmail.com, xiehous315@163.com, xh1987222@126.com, lylong@mails.tjmu.edu.cn, and liu_wq2002cn@yahoo.com.cn. Ji-long Shen, Department of Parasitology, Anhui Medical University, Hefei, People's Republic of China, E-mail: jlshen@ahmu.edu.cn. Xiao-hong Guan and Zhen-qing Feng, Key Laboratory of Antibody Technology of the Ministry of Health, Nanjing Medical University, Nanjing, People's Republic of China, E-mails: xhguan@163.com and fzhenqing@163.com. Meng-xing Xu, Institute of Schistosomiasis Control, Wuhan Center of Disease Control, Wuhan, People's Republic of China, E-mail: xfk@whcdc.org.

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