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Chicken egg yolk antibodies (IgY) for detecting circulating antigens of *Schistosoma japonicum*☆

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ABSTRACT

Background: IgY isolated from egg yolk has been widely used in immunodiagnostic tests, including tests to detect circulating antigen (soluble egg antigen or SEA) of *Schistosoma japonicum*.
Results: A sandwich ELISA was established using a combination of anti-*S. japonicum* SEA-IgY polyclonal antibodies and IgM monoclonal antibodies. To explore sensitivity and specificity of the sandwich ELISA, serum samples from 43 patients infected with *S. japonicum* were tested. All acute cases and 91.3% of the chronic cases showed a positive reaction. Only 5% of the control sera from healthy persons gave a positive response. Cross-reactions with antibodies to nine other parasites were rare.
Conclusion: The developed immunoassay is reasonably sensitive and specific. It could be used for field research and treatment efficacy assessments.

1. Background

Schistosomiasis as a zoonosis that remains one of the most prevalent parasitic infections in the world [1–3]. It has significant economic and public health impacts, including in the People's Republic of China (P.R. China) [4]. The distribution of schistosomiasis in the country has changed over the last 50 years; both the population infected and that at risk of infection have been reduced through extensive control programmes [5]. The total number of schistosomiasis patients in 2008 was 412,927, a decrease of 96.44% compared with the number in 1949 (11,612 million) [6]. While the transmission of schistosomiasis has been interrupted in Guangdong, Fujian and Zhejiang provinces as well as in Guangxi Zhuang autonomous region and Shanghai municipality, the disease is still prevalent in the lake areas of Hunan, Hubei, Jiangxi, Anhui and Jiangsu provinces as well as in some mountainous regions of Sichuan and Yunnan provinces (mountainous areas)[5]. In most endemic areas, the intensity of human infections is declining due to sustained control and prevention measures, and thus etiological and parasitological diagnosis is becoming more and more difficult [7].

The primary tool of schistosomiasis control currently is praziquantel-based chemotherapy, but there are fears that large-scale, repeated, single-dose chemotherapy could promote the development of drug resistance [8–10]. According to some reports, praziquantel-resistant strains of *S. mansoni* have emerged [11]. Therefore, it is necessary to explore ways to accurately determine infection status and narrow the target population for treatment, e.g. by using rapid, low-cost sero-epidemiological methods. Such an approach could also reduce costs and workload of running and sustaining a control program.

After decades of development and improvements, immunologic tests for the diagnosis of schistosomiasis have achieved high sensitivity, specificity and feasibility. Among the developed approaches, the detection of circulating antibodies against *Schistosoma* spp. is a low-cost, simple way and has become the most widely used approach in field surveys in P.R. China [12–15]. However, circulating antibodies tend to remain elevated for considerable time after treatment [16],

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particularly antibodies against soluble egg antigen (SEA), making it difficult to distinguish between past and current infections.

Recent research has focused on the identification of antibodies which disappear more rapidly following treatment, so-called short-life antibodies.IgG4 is one of them and was initially thought to have the potential to indicate treatment success [17]. However, tests focusing on IgG4 appeared to be less sensitive compared to those detecting total IgG. Another approach is to detect circulating antigens (CAg) which represent the schistosome parasites metabolic and secreted antigens in the patient's blood, saliva, urine or other body fluids. Detection of CAg thus is a more direct measure of the worms' presence and burden, providing accurate information on the status and intensity of infection. Moreover, CAg rapidly disappear following successful treatment, so that detection of circulating antigens can be used for treatment efficacy assessments. There are also some references of work done on *Schistosoma mansoni* and *Schistosoma heamatobium*, Van Dam GJ et al. [18] demonstrated a high sensitivity and association with the intensity of infection as measured both by egg counts, and by circulating anodic antigen and CCA levels determined by enzyme-linked immunosorbent assay. But because these two species were less important in China, we know little of the progress.

Egg yolk immunoglobulin (IgY), the predominant serum immunoglobulin in birds, reptiles and amphibians, has the same function as mammalian IgG. Additionally, IgY can be obtained from egg yolk easily and at high quantity [19,20]. IgY is stable and can be stored as long as 6 months at 4 °C. Moreover, IgY do not activate the mammalian complement system, and do not bind to mammalian rheumatoid factors, protein A or G. These qualities make the production of antibodies against conserved mammalian proteins more successful e.g. in chicken than in mammals. Based on these advantages, IgY has been widely used in veterinary science, functional food and bio-products, and has now been recognized as showing potential for human disease control and treatment applications [21–24].

In the present study, we attempted to develop an immunoassay for the detection of circulating antigens of *S. japonicum* based on the IgY technology. We produced polyclonal antibodies against soluble egg antigens (SEA) of *S. japonicum*, and monoclonal antibodies against a single epitope on a specific SEA-antigen. The two antibodies were then used to establish a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) for schistosomiasis *japonica* diagnosis in humans.

2. Methods and materials

2.1. SEA preparation

Seven New Zealand rabbits (Shanghai Laboratory Animal Center, Chinese Academy of Sciences) were each infected with 1 500 *S. japonicum cercariae*. The rabbits were sacrificed 42 days post infection, and schistosome eggs collected from the rabbits' liver. The SEA was prepared as follows: the lyophilized schistosome eggs were weighed and mixed with 1% physiological saline. The mixture was soaked at 4 °C for 4 days, interrupted by twice-daily vigorous mixing for 2 min. The mixture was then sonicated three times for 3 min each at an interval of 3 min, followed by centrifugation at 12,000×*g* for 30 min at 4 °C, discard the supernatant, the precipitation was the total proteins of a crude extract from eggs of *S. japonicum*. It was called soluble egg antigen (short for SEA). The concentration of SEA in the supernatant was determined with the Bradford reagent (Bio-Rad, USA) before storage at −20 °C pending usage.

2.2. Chicken immunization

Three 17-week-old egg-laying Hy-Line W-36 hens were kept in individual cages with ample food and water ad libitum throughout the course of the study. For immunization, 50 μg SEA was injected intravenously into the wing vein. Boosters of 50 μg SEA were administered once on day 14 post-immunization and then every 4 weeks for a total of 16 weeks (total 4 injections). Eggs were collected every day and marked with the date after the first immunization and stored at 4 °C pending further use.

2.3. Extraction of IgY from egg yolk

The Egg Stract IgY Purification System (Promega, USA) was used to extract IgY from egg yolk. The egg yolk was separated from the egg white with an egg separator, the yolk sac discarded and the egg yolk collected in a clean tared beaker. The weight of the yolk was measured and 1 g egg yolk was diluted with 3 volumes of precipitation solution A, mixed thoroughly for 5 min, and incubated for 5 min before centrifugation at 10,000×*g* for 15 min at 4 °C. The supernatant was then filtered through sterile gauze, and 1/3 volumes of Precipitation Solution B was slowly added while thoroughly stirring. The precipitate containing IgY was pelleted by centrifugation at 10,000×*g* for 15 min at 4 °C, re-suspended with 2 mL water, and dialyzed against PBS equal to the original volume of the egg yolk. The concentration of IgY was estimated with the Bradford reagent (Bio-Rad), and the solution stored at −20 °C.

2.4. Labeling of monoclonal antibody IgM with horseradish peroxidase (HRP)

Coupling of the IgM monoclonal antibody (this monoclonal antibody was against a single epitope on a specific SEA-antigen, and it was from our laboratory) with HRP (Toyobo, Osaka) was performed according to the method of Nakane and Kawaoi [25] with minor modifications.

2.5. Development of IgY–IgM sandwich ELISA

Each well of an ELISA plate was coated with anti-SEA IgY diluted in sodium carbonate buffer, pH 9.6, and the ELISA plate was stored at 4 °C overnight. The plate was then washed 3 times with PBS containing 0.05% Tween 20, followed by blocking with 10% skimmed milk (wt/vol) in PBS. 100 μL of SEA antigen (20 μg/mL) was added to the wells, and the plate incubated at 37 °C for 1 h. The plate was then washed 3 times with PBS containing 0.05% Tween 20, 100 μL of HRP-conjugated IgM monoclonal antibody was added, and the plate incubated at 37 °C for 1 h. After the plate was washed, TMB(3,3′,5,5′-Tetramethylbenzidine)–hydrogen peroxide substrate was added to each well, and the plate was incubated for 15 min at 37 °C in the dark. The reaction was stopped by adding 2 N sulfuric acid. Substrate conversion was monitored at 450 nm (A450). Positive and negative controls were set in every plate. Results determination: a positive sample and a negative sample that need to give a ratio of at least 2.1 (OD of the positive divided by OD of the negative, $P/N \geq 2.1$), furthermore the positive sample needs to generate an OD value of at least 0.4; and if in that case, the samples to be tested and the negative sample give a ratio of at least 2.1($S/N \geq 2.1$), and the sample generate an OD value of at least 0.4, the sample is considered positive, if not, will be negative. Orthogonal test [25] was used to determine the best concentration of coating antibody and optimal working concentration of IgM-HRP.

2.6. Serum samples

Serum samples from 43 patients were obtained from the Serum Library of the National Institute of Parasitic Diseases in Shanghai. All samples were collected from patients who had been excreting eggs of *S. japonicum* as confirmed by the Kato–Katz thick smear test [26] or the miracidial hatching test [27]. Among the patients, 20 had been defined as acute schistosomiasis cases and the others as chronic