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Evaluation on the applied value of the dot immunogold filtration assay (DIGFA) for rapid detection of anti-*Schistosoma japonicum* antibody

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Abstract

The dot immunogold filtration assay (DIGFA) is a rapid technique for the detection of anti-*Schistosoma japonicum* antibody. Its sensitivity with regard to sera obtained from patients with acute or chronic schistosomiasis was shown to be 100 and 96.9%, respectively. The specificity when using sera of people living in an area non-endemic for schistosomiasis japonica was 100%. Cross-reaction rates for paragonimiasis and clonorchiasis patients were 14.3% and 0%, respectively. Parallel serum tests of 1091 residents from an area endemic for *S. japonicum* by means of DIGFA, enzyme-linked immunosorbent assay and indirect haemagglutination test resulted in positive rates of 9.3%, 11.5% and 11.0%, respectively. Thus, there was a high level of agreement between the sets of results ($P > 0.05$). In conclusion, DIGFA holds considerable promise for rapid and accurate diagnosis of *S. japonicum*, as it does not require any specific instruments and can be applied with ease. DIGFA has therefore several advantages over conventional diagnostic approaches and is useful not only for screening and sero-epidemiological surveys in the field, but also in clinical settings.

Keywords: Schistosomiasis; *Schistosoma japonicum*; Diagnosis; Dot immunogold filtration assay; Antibody detection; Cross-reactivity

1. Introduction

Schistosomiasis japonica is still a serious public health problem in China because it is a danger to human health and an important factor holding back the social and economic development (Zhou et al., 2005). Traditional methods for diagnosing an

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infection with *Schistosoma japonicum* is pathogen identification, which includes parasite egg detection by light microscopy and the miracidium hatching test (Ross et al., 2001). Both methods, however, are labour-intensive, time-consuming, somewhat messy, and have a low sensitivity in patients with only light infection intensities (Wu, 2002). Large-scale use of the highly efficacious antischistosomal drug praziquantel in the national schistosomiasis control programme over the past 25 years resulted in a steady decrease of the overall endemicity leading to a lowering of the sensitivity of direct parasitological techniques (Chen, 2005; Zhou et al., 2005). Against this background, several immunodiagnostic methods have been developed. The intradermal test (ID), developed more than 50 years ago, was the first immunological approach applied to schistosomiasis japonica, using either schistosome eggs or adult worm antigens (Wu, 2002; Zhu, 2005). Subsequently, the circumoval precipitin test (COPT), the indirect haemagglutination test (IHA) and enzyme-linked immunosorbent assay (ELISA) have been developed and widely used to date (Zhu, 2005). However, despite their high sensitivity and specificity, these three techniques are time-consuming, and they require trained personnel and special instruments.

With the development of an immune labelling technique in the late 1980s, a rapid, colloid gold dot-binding assay, namely the dot immunogold filtration assay (DIGFA), has been developed and used successfully for detection of antibodies to HIV and alpha fetoprotein (Spielberg et al., 1989; Cao et al., 1991). Colloid gold is characterized by high-density electrons, which gather around corresponding ligands forming a red dot visible to the naked eye. At our institute, we have recently established the DIGFA technique for detection of anti-*S. japonicum* antibody (Ding et al., 1998; Shen et al., 2000).

Here, we report the results from an investigation carried out among different study groups consisting of people with various levels of infection intensities living in *S. japonicum*-endemic areas, as well as healthy controls from non-endemic areas. We assessed the diagnostic value of the DIGFA for detection of anti-*S. japonicum* antibody, and compared the results with those derived from ELISA and IHA techniques. We also examined the cross-reactivity with food-borne trematodes and soil-transmitted helminths.

2. Materials and methods

2.1. Soluble egg antigen of S. japonicum

The ova of *S. japonicum* were collected from liver tissue of artificially infected rabbits. After repeated washing, the purified ova were ground into a fine powder using mortar and, subsequently, this powder was diluted with normal saline to make a 1% soluble egg antigen (SEA) solution. After alternate lyophilization at temperatures of −20 and 37 °C for three rounds, the solution was kept for 3 days at a temperature of 4 °C. The solution was ultrasonically disintegrated in ice water for three times, 2 min each. After 30 min centrifugation at 4000 rpm, and an additional 20 min at 12,000 rpm, a clear liquid with a protein content of 15 mg/ml appeared which was stored at −20 °C.

2.2. Serum samples

Sera from 60 cases with acute schistosomiasis japonica upon clinical diagnosis, and 161 chronic cases, as diagnosed by *S. japonicum* eggs in their stool samples were collected from an endemic area in Jiangxi province. Sera of 135 healthy people were collected from Yueyang city, Hunan province. Sera from 1091 residents living in an endemic area were collected from Guichi city and Dongzhi county, Anhui province. Sera of 123 people from a non-endemic area were collected from Zhoushan city, Zhejiang province. Sera were also collected from 154 healthy people living in Hangzhou city when they presented for routine health check-ups.

In addition, sera were collected from patients with food-borne trematodiasis (i.e. 35 cases with paragonimiasis and 20 cases with clonorchiasis) and soil-transmitted helminthiasis (i.e. 30 cases with a hookworm infection and 30 patients infected with *Ascaris lumbricoides*). These 115 sera were all provided by the Institute of Parasitic Diseases, Zhejiang Academy of Medical Sciences (Hangzhou, China).

2.3. DIGFA

As a first step, 2 μl (about 30 μg) of *S. japonicum* SEA were added onto a nitrocellulose membrane (aperture: 0.65 μm) and allowed to dry at room temperature. After the preparation of the solid phase antigen membrane, it was preserved at 4 °C.

In a second step, the colloid gold labelled binding product was prepared as follows. Rabbit anti-human IgG was separated and purified by means of octanoic acid (Xu and Tao, 1989), with the titre of IgG exceeding 1:64 by double diffusion. Orange colour colloid gold solution was obtained according to the trisodium citrate deoxidation method (Shen et al., 1999). The pH was adjusted to 8.2 with 0.1 mol/l potassium carbonate. IgG was added to colloidal gold solution until it became red. Then polyethylene glycoll (PEG) was added to the solution until a final concentration of 0.5 mg/ml was reached. Finally, purification was done by means of ultra centrifugation at 19,700 rpm for 30 min.

In a third step, kits were assembled by packing 20 test kits together consisting of a base and a tightly fitting lid with a 10 mm diameter opening in the middle. Under the opening a micropore solid phase antigen membrane of 25 mm diameter was positioned on top of a water-absorbing pad. Each diagnostic kit contained one flask of colloid gold-labelled antibody and two flasks of washing solution. Each diagnostic kit was equipped with two quality-control tests (Shen et al., 1999).

The tests were done as follows. First, one drop of solution B (pH 8.2 Tris–HCl solution) was put on the kit. Second, 50 μl of serum were added. Third, another drop of solution B was added. Fourth, one drop of solution A (rabbit anti-human IgG probe labelled by colloidal gold) was added. Finally, the nitrocellulose membrane was washed in the centre of the kit with two drops of solution B (Shen et al., 1999).

The result can be read with the naked eye comparing the resulting colour with that of the controls. When the colour of the dot is similar to that of the enclosed quality-control, but somewhat lighter, then the serum is considered positive and marked 1+. When the resulting colour is equal to that of the quality control, the serum is also positive, but marked with 2+. A resulting colour, which is deeper than the control is marked 3+. In cases the resulting colour is only pale or appears with a pink background it is considered negative (−).

2.4. ELISA

The ELISA test was carried out according to a standard procedure (Zhang et al., 1990). In brief, Embed 96-well reaction plates with 100 μl of 1:700 diluted *S. japonicum* SEA were stored overnight. The sera were diluted by a factor 200 before testing. Horseradish peroxidase labelled SPA was prepared at our institute, with a dilution of 1:30,000, using TMB as base. We employed a stop reaction with 2 M H_2SO_4, and measured the optical density (OD) value with an ELISA instrument at a wavelength of 490 nm. According to the manufacturer's instructions, positive and negative control sera were measured simultaneously. In cases where the average OD value of double wells was 2.1-fold higher than that of the negative control sera, it was regarded as a positive reaction.

2.5. IHA

An IHA test kit was provided by the Anhui Provincial Institute of Schistosomiasis Control. The test was carried out according to Wu et al. (1991). In brief, 100 μl of normal saline was placed into the first well of the transverse line, while 25 μl were placed into wells 2 and 3. Then, 25 μl of serum were added to the first well and thoroughly mixed. In a subsequent step, 25 μl of this solution was added to the second well and mixed as before. The same procedure was repeated for the third well. Hence, the concentrations in the second and third wells were 1:10 and 1:20, respectively. Known positive and negative control sera were tested simultaneously on each plate.

We adhered to the following procedure. One drop of 2.5% sensitized red blood cell was placed into each well, shaken and then kept at room temperature for 1 hour. Observations were made by the naked eye. The highest titre where agglutination still appeared was regarded as the terminal point of a positive reaction. If a positive reaction appeared at a titre $\geq$1:10, the serological test was considered to be positive.

3. Results

3.1. Preliminary testing of the sensitivity of DIGFA when applied for detection of anti-S. japonicum antibody

In a first preliminary test, five sera from patients infected with *S. japonicum* who had an infection intensity of 196 eggs/g of stool (epg), and five sera from non-infected control people were tested by the DIGFA method. All tests were performed three times. In all cases the sera from egg-positive patients showed posi-

Table 1
Detection of anti-*S. japonicum* antibody using DIGFA in various cohorts of subjects

Sources of sera	No. of cases	No. of positives	Positivity rate (%)
Acute schistosomiasis	60	60	100.0
Chronic schistosomiasis	161	156	96.9
Healthy people in endemic area	135	33	24.4
People in non-endemic area	123	0	0
Paragonimiasis	35	5	14.3
Clonorchiasis	20	0	0

tive results, while those from healthy controls exhibited negative results.

3.2. Sensitivity and specificity of DIGFA

Encouraged by these preliminary findings, we extended the application of the DIGFA method to a larger number of sera. Table 1 summarises the results of the DIGFA technique for detection of anti-*S. japonicum* antibody. The sensitivity in detecting patients with either acute or chronic schistosomiasis japonica was 100% (60/60) and 96.9% (156/161), respectively. The positivity rate for sera obtained from healthy controls living in an area endemic for *S. japonicum* was 24.4% (33/135), and the corresponding percentage for people living in non-endemic areas was 0% (0/123). The cross-reaction rates for paragonimiasis and clonorchiasis patients were 14.3% (5/35) and 0% (0/20), respectively.

3.3. Parallel experiments using three different assays for the detection of anti-S. japonicum antibody in residents of endemic areas

The positivity rates for detection of anti-*S. japonicum* antibody in sera obtained from residents living in a schistosome-endemic area, using DIGFA, ELISA and IHA, were 9.3% (102/1091), 11.5% (125/1083) and 11.0% (120/1091), respectively (Table 2). Statistical analyses, using a χ^2 test, revealed no significant differences between the three methods.

Table 2
Detection of anti-*S. japonicum* antibody by means of three different assays

Technique	No. of cases	No. of positives	Positivity rate (%)
DIGFA	1091	102	9.3
ELISA	1083	125	11.5
IHA	1091	120	11.0

DIGFA vs. ELISA: $P = 0.095$; DIGFA vs. IHA: $P = 0.202$; ELISA vs. IHA: $P = 0.689$.

3.4. Parallel experiments using three different assays for detection of anti-S. japonicum antibody in sera of healthy controls and patients infected with other parasites

The positivity rates for detection of anti-*S. japonicum* antibody in sera of healthy controls by DIGFA, ELISA and IHA were 0.6% (1/154), 3.2% (5/154) and 3.2% (5/154), respectively (Table 3). χ^2 test statistics revealed no significant differences between the three methods ($P > 0.05$). Table 3 also summarizes the cross-reaction rates with other parasitic infections, namely *Paragonimus* spp., hookworms and *A. lumbricoides*. The cross-reaction rates for detection of anti-*S. japonicum* antibody in sera of paragonimiasis patients by the three assays were 7.7% (1/13), 0% (0/13) and 0% (0/13), respectively. The corresponding percentages for patients infected with hookworms were 0% (0/30), 6.7% (2/30) and 3.3% (1/30), respectively. A cross-reaction rate of 6.7% (2/30) was found for all the sera obtained from patients with an *A. lumbricoides* infection irrespective of the test employed.

Table 3
Detection of anti-*S. japonicum* antibody using three different assays in various cohorts of subjects

Population group	No. of people	DIGFA		ELISA		IHA	
		No. of positive	Positivity rate (%)	No. of positive	Positivity rate (%)	No. of positive	Positivity rate (%)
Healthy people	154	1	0.6	5	3.2	5	3.2
Patients with paragonimiasis	13	1	7.7	0	0	0	0
Patients with hookworm infection	30	0	0	2	6.7	1	3.3
Patients with *A. lumbricoides* infection	30	2	6.7	2	6.7	2	6.7

4. Discussion

In order to develop a rapid and simple immunodiagnostic assay for schistosomiasis japonica, colloid gold was conjugated with rabbit anti-human IgG, so that antibodies in the sera of schistosomiasis patients could be detected. The results showed a 100% sensitivity of the DIGFA among 60 cases of acute schistosomiasis, whereas the sensitivity among 161 cases of chronic schistosomiasis was only slightly lower, namely 96.9%. Colloid gold is characterized by high-density electrons, which gather around corresponding ligands forming a red dot. The strong contrast offered by the colloid gold marker facilitates reading of the test and thus further increases sensitivity. The specificity of the assay was 100% among 123 people living in an area non-endemic for *S. japonicum*, 85.7% among 35 cases with paragonimiasis and 100% among 20 cases with clonorchiasis. It is important to note that the technique, on top of being rapid and simple to operate, does not require any specific instrument, is highly reliable as repeated tests revealed the same results, and the reagent is stable at 4 °C for at least 6 months (Ding et al., 1998).

In a field evaluation of the DIGFA, results of a parallel experiment for detection of anti-*S. japonicum* antibody by either DIGFA, ELISA or IHA among residents living in a *S. japonicum*-endemic area demonstrate that the positive rates were 9.3%, 11.5% and 11.0%, respectively and the differences obtained between the three techniques show no statistically significant difference. The positivity rates for detection of anti-*S. japonicum* antibody in sera of healthy people by DIGFA, ELISA and IHA were 0.6%, 3.2% and 3.2%, respectively, and hence there was no statistical differences between the three methods. The cross-reactivities for detection of anti-*S. japonicum* antibody in sera obtained from patients with either paragonimiasis, hookworms or *A. lumbricoides* by the three assays were 4.1%, 5.5% and 4.1%, respectively. Appropriate statistical tests revealed no significant differences between the three methods. The DIGFA thus has a high specificity. Its results are available within 2–3 min, whereas IHA and ELISA need about 90–120 min. Importantly the results from the DIGFA can be preserved for control at a later time, so this approach holds promise for diagnosis and sero-epidemiological surveys, especially for rapid clinical diagnosis and community-based surveys.

In conclusion, we have established the DIGFA technique for detection of anti-*S. japonicum* antibody and developed the kit over a 2-year period commencing in 1998 (Ding et al., 1998; Shen et al., 2000). Thus far, some 200,000 cases have been examined in clinical settings and diagnosed at local anti-schistosomiasis control stations and during sero-epidemiological surveys carried out in the provinces of Anhui, Guangdong, Jiangxi and Zhejiang and Shanghai city. In view of DIGFA exhibiting a similar sensitivity and specificity as both ELISA and IHA, the test holds promise for further large-scale application. The only shortcoming of the DIGFA is that the results it generates are only qualitative. We currently focus on further developing this method so that results could be analysed in a semi-quantitative or fully quantitative manner (Chen and Wen, 2005).

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