

## **A Monoclonal Antibody-based Dot Immunogold Filtration Assay: A Rapid Field Applicable Technique for Immunodiagnosis of Active Schistosomiasis**

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**Abstract:** A simple and rapid dot-immunogold filtration assay (DIGFA) technique was used in the present study for rapid detection of antigenaemia in the sera of schistosomiasis patients, using a pair of monoclonal antibodies, the first as antigen capture and the second labeled with red color colloidal gold as antigen detector. Sandwich – ELISA technique, dot-ELISA and dipstick ELISA were used in comparison with the DIGFA technique to evaluate its sensitivity and specificity. The results showed that the percentage positivity of antigenaemia in schistosomiasis patients' sera, using sandwich-ELISA technique was 100%, while in dot-ELISA and dipstick-ELISA the positivity rate was 89.7% and 91.2% respectively. The DIGFA technique showed 97.1% positivity, while the sensitivity and specificity of DIGFA were 95.8% and 95% respectively. In sandwich-ELISA, dot-ELISA and dipstick-ELISA the sensitivity was 100%, 90.7% and 91.9% respectively, and the specificity was 96.6%, 91.9% and 91.9% respectively. A significant differences was observed between the results of DIGFA in schistosomiasis patients compared with other techniques and the healthy control group ( $p < 0.01$ ). Therefore, in conclusion, DIGFA is a method that has many advantages over conventional diagnostic techniques by being simple, rapid, sensitive, specific and reliable method without expensive equipments. So, it could be applied with a broad prospect and for mass diagnosis and epidemiological survey protocols.

**Key words:** Immunogold, monoclonal antibodies, Dot-ELISA.

### **INTRODUCTION**

Schistosomiasis is one of the most widespread parasitic infections. The incidence of the disease is very high in developing countries, especially in poor hygiene area (Zhou *et al.*, 2005). In Egypt *Schistosoma mansoni* and *Schistosoma haematobium* cause intestinal and urinary schistosomiasis respectively, and still a serious public health problem and an important factor holding back the social and economic development (El Ridi *et al.*, 1998). Traditional methods for diagnosing schistosomiasis infection are generally performed by fecal parasitological examinations such as Kato-Katz technique (Katz *et al.*, 1972). and miracidium hatching test (Ross *et al.*, 2001). Nevertheless, these methods have poor diagnostic efficiency when applied to individuals with low worm burden (Wen *et al.*, 2005). Hence, alternative laboratory diagnostic methods with evaluated high rates of sensitivity and specificity have been developed, using either schistosome eggs or adult worm antigens. Subsequently, circumoval precipitation test (COPT), indirect haemagglutination test (IHT) and enzyme-linked immunosorbent assay (ELISA) have been developed and widely used to date<sup>(1)</sup>. However, despite their high sensitivity and specificity, these three techniques are time-consuming and they require trained personnel and special instruments (Yong *et al.*, 2005).

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 (Cao *et al.*, 1991), anti-HAV IgM (Wei *et al.*, 1999), anti-HAV IgG (Shao *et al.*, 2003), anti-WSSV (white spot syndrome virus) of shrimp (Wang *et al.*, 2006) and anti-*Schistosoma japonicum* antibody (Yong *et al.*, 2005).

The present study aimed to evaluate the active infection of schistosomiasis *mansoni* by DIGFA technique using a pair of monoclonal antibodies raised against *S. mansoni* soluble egg antigen (SEA).

**Subjects and Methods:**

**Study population:**

A total of 125 subjects were included in the present study. The study included 68 patients infected with *Schistosoma mansoni*, 39 patients with other parasites including 20 with fascioliasis and 19 with echinococcosis and 18 healthy controls. All subjects were clinically diagnosed at out patients of TBRI clinic. Also, parasitological examination was performed.

**Parasitological Examination:**

Fecal samples were collected from each individual and examined for the presence of any parasitic infection by egg determination using *KatoKatz* method (Katz *et al.*, 1972).

**Serum Samples Collection:**

Sera from all cases infected with *Schistosoma mansoni*, other parasites and healthy controls were collected after parasitological and clinical examination and kept frozen at  $-20^{\circ}\text{C}$  till use.

**Production of Anti-s. Mansoni Monoclonal Antibodies (MABS):**

The production of MAb against *S.mansoni SEA* was carried out at the Department of Immunology at TBRI using hybridoma technology (Galfré & Milstien, 1981). A frozen cell line from different colonies was thawed rapidly and carefully transferred to 15 ml sterile tubes, centrifuged and the suspended cells were cultured in sterile 24 well plates with (RPMI) 1640 growth media (Sigma). Growing cultures were transferred into 50 ml flasks. Large amounts of MAb were propagated by growing cells as ascites tumors in BALB/c mice which exposed to ascetic drainage after abdominal swelling.

A pair of the produced MAb with high reactivity were chosen, isotype determined (Rockville, 1984), purified (Langone, 1982) and their chemical nature was determined to define monoclonal epitopes using periodate oxidation method (Woodward *et al.*, 1985) and trichloroacetic acid (TCA) method (De-Jong *et al.*, 1988). One of the MAb was peroxidase labeled (Tijssen & Kurstak, 1984) to detect antigen and the other was used as antigen capture. The two MAb produce were used in detecting schistosomal antigen in patients sera by different immunodiagnostic techniques, after detecting the optimum dilutions used for each technique.

**Immunodiagnosis Using Sandwich-ELISA (Voller *et al.*, 1974):**

Flat bottomed 96-microtiter plates were coated with antigen capture MAb (3 $\mu\text{g}$ /well), kept overnight at  $4^{\circ}\text{C}$ , then washed and blocked for 1 hour using 2.5% fetal calf serum.

Plates then, washed and sera were applied undiluted (100  $\mu\text{l}$ /well), then incubated 1 h at room temperature, then washed and 100  $\mu\text{l}$ /well of peroxidase labeled MAb (1:1000 dilution) was added to the plate and incubated 2 h at room temperature. Plates were washed, then orthophenyline diamine (OPD) substrate was added (diluted in 0.05 M phosphate citrate buffer with urea hydrogen peroxide). Reactivity was estimated spectrophotometrically at 492 nm after adding 8 N  $\text{H}_2\text{SO}_4$  (50  $\mu\text{l}$ /well) to stop the reaction.

**Immunodiagnosis Using Dot-ELISA (Boctor *et al.*, 1987):**

The technique was performed using Bio-Rad apparatus which include 96-wells and attached to vacuum source. Nitrocellulose membrane sheet (NC) was used as capture matrix after wetting with PBS buffer. The NC membrane was kept between pre-wetted two filter paper sheets and put into the apparatus, then the coating MAb was applied (1  $\mu\text{g}$ /well), incubated for 30 min, then washed with washing buffer (PBS/T), blocked for 15 min with 2.5% FCS, then washed and sera were applied (diluted v/v with blocking buffer) and incubated for 30 min. The sera were removed and washed, then peroxidase labeled mAb was added at dilution (1:200), then incubated for 15 min and washed, then NC membrane was removed from apparatus to a tray including PBS buffer. The 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate was freshly prepared in dark by dissolving in urea hydrogen peroxide and 0.05 M phosphate citrate buffer. The reaction was stopped, just after color development, with cold deionized DW.

**Immunodiagnosis Using Dipstick ELISA (Zhu *et al.*, 2005):**

Dipstick is a modified dot-ELISA and Bio-Rad was also used as previously described in dot ELISA technique, but the upper part of apparatus having only 48 longitudinal wells instead of dots. After coating NC membrane with coating MAb, then blocking with 2.55 FCS, the NC membrane was removed, then cut into longitudinal strips, each with 0.5 cm width and 9 cm length. Each strip was re-adhered to another adhesive membrane to get separate longitudinal strips. The previous technique used in dot-ELISA was continued as mentioned before using the prepared strips.

**Immunodiagnosis Using DIGFA Technique (Shen et al., 1999):**

The dispersion of colloidal gold particles was prepared. To make 100ml of colloidal gold solution, two stock solutions were prepared. Solution (A): 1 ml 0.01% Au Cl<sub>3</sub>-HCl-4H<sub>2</sub>O were dissolved in 80 ml D.W. Solution (B): 4 ml 0.1% tri-sodium citrate. 2H<sub>2</sub>O dissolved in 16 ml D.W. Solution A,B were mixed till red color was formed , then solution was warmed up to 95°C then cooled on ice and kept at 4°C till use.

The gold-labelled anti-*S. mansoni* SEA MAb conjugate was prepared with some modifications (Qiao et al., 1996). The pH of the colloidal gold solution was adjusted to 8.3, then the purified anti-*S. mansoni* MAb was added to the solution with stirring. The amount of MAb mixed with gold solution was evaluated (Wen et al., 2005), then accordingly, 130 ul of MAb were added to 10 ml colloidal gold solution and the reaction was allowed to proceed for 10 min with slow stirring , and then overnight at 4°C without stirring. The reaction mixture was centrifuged at 2500 r.p.m. , for 5 min. The supernatant was discarded and the precipitate was dissolved in 1 ml 0.01 M PBS (pH 7.4) containing 2.5% FCS, thus forming the colloidal gold probe, then it was kept at 4°C till use.

**Preparation of the Immune Filtration Device:**

A filtration device was manufactured according to the design of the study group. It was consisted of a small plastic box (2cm x 2cm x 2cm) with a test hole 10mm in the center. The box was filled with water-absorbing material, and NCM was laid on the top of this material in such a manner that the reaction deposit between antigen and labeled MAb, would be visible through the opening (fig 3 ).

**Application of the Technique (Wang et al., 2006):**

The NCM was soaked by D.W , then coated with MAb and kept overnight at 4°C. After washing, NCM was blocked for 10 min at room temperature using 2.5% FCS, then washed, dried, cut into (1 cm x 1 cm) cubes and fixed into the immune filtration device. NC membrane was activated with one drop of PBS/T buffer, then 5 ul of serum was dripped slowly to the center of the device test hole. After 1 min, the serum was washed with 2-3 drops of washing solution (PBS). After washing, 30 ul of the colloidal gold probe was added, for 1 min, then washed with 2-3 drops of washing solution . The appearance of a reddish dot in the hole opening indicated a positive reaction, while the absence of such a dot was an indication of a negative reaction.

**Statistical Analysis:**

All values were evaluated for the significance of differences between studied groups and control group by student "t" test. Differences between negative controls and test groups were considered significant at p< 0.05.

## RESULTS AND DISCUSSION

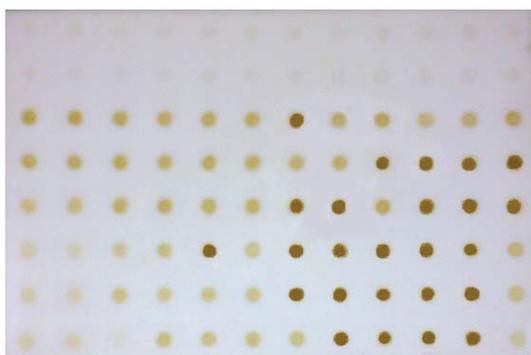
**Determination of Circulating *S. mansoni* SEA in Sera of Patients:**

**Sandwich ELISA:**

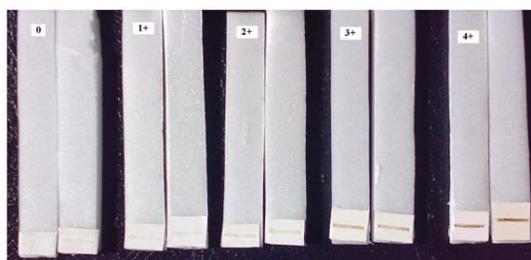
The level of circulating *S. mansoni* SEA detected by ELISA was estimated as OD readings at 492 nm. Cut off value for positivity was calculated as mean + 2 SD. The rate of positivity in *S. mansoni* infected group using sandwich ELISA technique was 100%, while other parasite group (fascioliasis and echinococcosis) showed 5.1% false positivity. The sensitivity of the technique was 100% and the specificity was 96.6%, while the diagnostic efficacy was 98.3%. According to the previous results, a very high significant differences between mean OD readings of sera of schistosomiasis patients compared to healthy control group was detected (p<0.001).

**Dot-ELISA:**

Using Dot-ELISA method for detection of circulating *S. mansoni* SEA in sera of schistosomiasis group, the color intensity was illustrated in table (2). Data obtained by using Dot-ELISA showed that 61 cases out of 68 cases were positive (89.7%), while 7 cases gave false negativity. A cross reactivity of other parasites group (*Fasciola hepatica* and *Echinococcus granulosus*) evaluated by Dot-ELISA showed a percentage of 12.8% (table 2). The color intensity scores corresponding to grade of positivity and grade of negativity for circulating *S. mansoni* SEA detection in sera of schistosomiasis and different studied groups are shown in fig (1). The sensitivity of dot-ELISA method was 90.7%, the specificity was 91.9% and the diagnostic efficacy was 91.3%. A high significant difference between schistosomiasis group and healthy control group was detected (p < 0.001).



**Fig. 1:** Application of patients' sera for the detection of circulating *S. mansoni* SEA using Dot-ELISA.



**Fig. 2:** Application of patients' sera for the detection of circulating *S. mansoni* SEA using Dipstick-ELISA.

**Dipstick-ELISA:**

Using Dipstick-ELISA for detection of circulating *S. mansoni* SEA in sera of schistosomiasis group, the data obtained showed that 62 out of 68 total cases were positive (91.2%), while 6 cases gave false negative results. The other parasite group including *Fasciola hepatica* and *Echinococcus granulosus*, gave false positive reaction equal to 12.8% (table 2). The color intensity grades was illustrated in fig (2). The sensitivity of the technique was 91.9%, while specificity was 91.9% and the diagnostic efficacy was 91.9%. A high significant correlation was found between schistosomiasis group and healthy control group. ( $p < 0.001$ ).

**Table 1:** Species Incidence of positivity for specific antigen detection in sera of different parasitic groups using Conventional ELISA, Dot- ELISA, Dipstick- ELISA and DIGFA.

Groups	No. of cases	Sandwich-ELISA		Dot- ELISA		Dipstick- ELISA		DIGFA	
		No. of positives	Positivity %						
Schistosomiasis	68	68	100%	61	89.7%	62	91.2%	66	97.1%
Fascioliasis	20	1	5%	3	15%	3	15%	2	10%
Echinococcosis	19	1	5.3%	2	10.5%	2	10.5%	1	5.3%
O.P.	39	2	5.1%	5	12.8%	5	12.8%	3	7.7%
Negative control	18	0	0	0	0	0	0	0	0

O.P.: Other parasites.



**Fig. 3:** A negative and a positive sample for application of patients' sera for the detection of circulating *S. mansoni* SEA using DIGFA.

**Table 2:** The color intensity grades for circulating *S. mansoni* SEA in sera of schistosomiasis, fascioliasis, echinococcosis and negative control detected by using Dot- ELISA, Dipstick- ELISA and DIGFA.

Groups	No. of cases	Dot- ELISA					Dipstick- ELISA					DIGFA				
		Visual grades					Visual grades					Visual grades				
		0	1+	2+	3+	4+	0	1+	2+	3+	4+	0	1+	2+	3+	4+
Schistosomiasis	68	0	7	11	26	24	0	6	15	22	24	0	2	12	25	29
Fascioliasis	20	16	1	3	0	0	15	2	3	0	0	17	1	2	0	0
Echinococcosis	19	15	2	2	0	0	16	1	2	0	0	17	1	1	0	0
Negative control	18	17	1	0	0	0	17	1	0	0	0	16	2	0	0	0

Visual grades (0, 1) are considered negative while visual grades (>1+) are considered positive.

**Table 3.**

Technique	Sensitivity %	Specificity %	Efficacy %
Sandwich- ELISA	100%	96.6%	98.3%
Dot- ELISA	90.7%	91.9%	91.3%
Dipstick- ELISA	91.9%	91.9%	91.9%
DIGFA	95.8%	95%	95.2%

**DIGFA:**

Using DIGFA technique for detection of circulating *S. mansoni* SEA in sera of schistosomiasis group, the results obtained showed that 66 cases out of 68 cases were positive (97.1%) and 2 cases were negative. The rate of false positivity of other parasites group including *Fasciola hepatica* and *Echinococcus granulosus*, was 7.7% (table 1). The color intensity grades are shown in fig (3) and table (2). The sensitivity of DIGFA technique was 95.8% and the specificity was 95%. The diagnostic efficacy was 95.2%. A very high significant difference ( $p < 0.001$ ) was found between schistosomiasis group and healthy control group.

**Discussion:**

DIGFA is a new technique of solid phase labeled immunoassay in which NCM was used as the support and colloidal gold as the label. It is a rapid method, requiring about 5 minutes to be performed. Additionally, it is simple enough to be used by paramedical personnel without special training and allow reliable measurement of any specific antigen from a small volume of serum (10 µl) without using special equipments (Wei *et al.*, 1999; Xu *et al.*, 2006).

Using NCM in dot-ELISA, dipstick-ELISA or DIGFA techniques, it was found that 0.45 µm pore size of NCM was the optimal size to be used. It was mentioned that the pore size of the membrane affected the filtration rate of both MAb and serum, since with smaller pore size, the filtration rate was low, but if the pore size was too large, less antigen would be adsorbed on NCM, leading to decreased sensitivity of the assay (Qiaojia *et al.*, 1996). Also, before applying the DIGFA method, the patients sera must avoid any fibrin clots or any lipids because it might interfere with the antigen-antibody reaction leading to false positive or false negative results (Wei *et al.*, 1999). It was mentioned that the filtration device and the use of visible colloidal gold particles-conjugated instead of enzyme-conjugated in ELISA, ensures that DIGFA has many advantages. First, the reaction in DIGFA avoids possible interference by endogenous enzymes in ELISA (Wang *et al.*, 2006). This eliminates false positive results in DIGFA. Second, DIGFA is so efficient that the test can be completed in 3-5 minutes without any equipment or incubation. The third advantage is that DIGFA can avoid the pollution of poisonous or carcinogenic reagents used in ELISA such as diaminobenzidine (DAB) or orthophenylenediamine (OPD) (Shao *et al.*, 2003).

In the present study, the sensitivity and specificity of DIGFA technique in sera of schistosomiasis patients were slightly lower when compared to sandwich-ELISA. However, there was no significant difference in specificity of both assays. The same results were reached in different applications of both DIGFA and sandwich-ELISA (Qiaojia 1996; Wei *et al.*, 1999; Shao *et al.*, 2003; Yong *et al.*, 2005; Wang *et al.*, 2006; Xu *et al.*, 2006).

In brief, the present study showed that DIGFA technique is exhibiting a similar sensitivity and specificity as sandwich-ELISA and more than dot-ELISA and dipstick-ELISA and holds for further large-scale application for detecting different parasitic infections. It is rapid and simple procedure with clear visual interpretation of results that make it particularly suitable for field testing and epidemiological surveys. The only shortcoming of DIGFA is that the results it generates are only qualitative, even very clear. So, the further developing of the method must be focused on analyzing a semi-quantitative or fully quantitative manner.

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