

Anti-*S. mansoni* MAb-based Latex Agglutination: A reliable field applicable immunodiagnostic test for screening of active Human Schistosomiasis

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Abstract: Schistosomiasis is a major public health problem with a worldwide distribution. Diagnosis of this disease by simple and rapid immunoassays is a priority. The objective of the present study was to standardize and evaluate the latex agglutination test (LAT) as a simple test for the detection of circulating schistosomal antigen (CSA) in serum and urine samples of *S. mansoni* patients and compare it with ELISA. According to stool examination this study included 70 *S. mansoni* infected patients, 32 other parasites infected patients and 30 negative control samples. Characterization of MAb 12D/10F was done using several techniques including: ELISA, immunoelectrophoresis, polyacrylamide gel electrophoresis and immunoblotting as well as periodate and trichloroacetic acid treatment of target antigen for identification of its chemical nature. A polystyrene latex (0.81 μm) suspension was used as a carrier particle for anti-*S. mansoni* adult worm tegumental antigen monoclonal antibody (12D/10F) in the test. The Latex particles sensitized with MAb were used for the detection of CSA in urine and serum samples. The sensitivity of LAT assay was 90% in urine and 87.1% in sera versus 92.9% and 95.7% for ELISA. The specificity of LAT assay was 88.7% and 93.5% for urine and sera versus 87.1% and 93.6% for ELISA. The diagnostic efficacy of LAT was 89.1% and 90.2% for urine and serum samples, respectively versus 90.2% and 94.7% for ELISA. Moreover, a positive correlation was found between ova count in stool of *S. mansoni* infected patients and both the intensity of LAT and OD readings of ELISA in urine ($r=0.922$; $p<0.001$ and $r=0.865$; $p<0.001$, respectively) and in serum ($r=0.847$; $p<0.001$ and $r=0.781$; $p<0.001$, respectively). In conclusion, LAT is a suitable applicable diagnostic method in field survey especially when followed by ELISA as a confirmatory test in query false negative results. In the same time, more trials are required to increase the sensitivity and specificity of LAT to allow its use on a large scale in field surveys and as diagnostic kits for multiple parasitic infections. [Journal of American Science 2010;6(5):19-27]. (ISSN: 1545-1003).

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1. Introduction

Diagnosis of schistosomiasis is usually based on the microscopic detection of eggs in stool and urine samples. These methods showed high specificity and low sensitivity especially in light infection with the presence of daily variation phenomenon (De Vlas and Gryseels, 1992). Immunodiagnostic assays have been developed and used for the detection of specific antibodies in serum. However these assays cannot differentiate between recent and past infection and has the problem of cross reactivity among different helminthic parasitic infections (Mott and Dixon, 1982).

Antigen detection assays represent a useful alternative diagnostic tool in two respects. Firstly, the high sensitivity of the assays allowing diagnosis of active infection (Deelder *et al.*, 1989; 1994; Gryseels *et al.*, 1994; Demerdash *et al.*, 1995; Hanallah *et al.*, 2003); secondly, antigen assays allow direct measurement of worm burden than quantitative parasitological techniques that would be extremely valuable for immuno-epidemiological studies, ranging from transmission dynamics over

morbidity to immune responses and vaccine trials. Moreover, circulating antigen assays could be modified to be easy performed and field applicable. They are widely used for diagnosis of *Schistosoma* infection (WHO, 2000). However, up till now it is not introduced for community diagnosis of schistosomiasis in Egypt.

The use of monoclonal antibodies (MAbs) has greatly increased the sensitivity and specificity of assays for detection of circulating schistosomal antigen (CSA) (Demerdash *et al.*, 1995). Tegumental antigens develop within 3 hours of host penetration by cercariae and thus their detection would diagnose active *S. mansoni* infection very early and reflect worm burden (Davis, 1986) and proved to be an efficient immunodiagnostic tool for schistosomiasis (Hanallah *et al.*, 2003).

The development of simple, rapid and sensitive methods for detecting CSA are needed as most available assays require several laboratory equipment and highly skilled persons. Being simple and rapid; latex-based diagnostic tests have been

used for detecting specific antigens or antibodies in several diseases (Bangs, 1990).

This study aimed at the development of MAb-based LAT agglutination test as a simple, rapid and field applicable screening test for CSA in urine and serum samples of human schistosomiasis.

2. Material and Methods

Parasitological Examination

This work was conducted on 70 *S. mansoni* infected patients, 32 patients harboring other parasites than *Schistosoma* [*Fasciola* (n= 15), *Echinococcus granulosus* (n= 10) and *H. nana* (n= 7)] and 30 healthy individuals. All patients were subjected to the following investigations: stool examination using merthiolate iodine formaldehyde-concentration (MIFC) method for detection of all helminth eggs and protozoal cysts. Three slides were prepared for egg count of schistosome eggs in stool using Kato-thick smear technique (Siongok *et al.*, 1976). Blood samples were collected from all cases and sera were separated, aliquoted and kept at -70° C until used. According to the egg count per gram faeces, schistosomiasis group was classified into high (> 300 eggs/g faeces), moderate (100-299 eggs/g faeces) and light (<100 eggs/g faeces) subgroups according to their intensity of infection.

Collection and processing of urine

Patients and healthy subjects (negative controls) were asked to provide freshly voided urine in 20 ml test tubes, and 1 ml of urine was transferred to an eppendorf (Hamburg, Germany) tube. The tubes were put into a tube holder and placed into a boiling water bath for five minutes, and then allowed to cool to ambient temperature before conducting the test.

Preparation of adult worm tegumental antigen

Viable *S. mansoni* adult worms were purchased from the Schistosome Biological Supply Program at Theodor Bilharz Research Institute, Giza, Egypt. The *S. mansoni* adult worm tegumental antigen (Sm AWTA) was prepared from living worms according to Oaks *et al.* (1981).

Monoclonal antibody production

Spleen cells from BALB/c mice immunized with Sm AWTA were fused with non-secreting murine myeloma cells (P3 X 63 Ag. 8). Immunization was performed according to Cianfriglia *et al.* (1983). Fusion was performed in the presence of 43% polyethylene glycol (Sigma) as modified from Galfre and Milstein (1981).

Hybridomas were screened for anti-Sm AWTA antibodies by ELISA. Hybrids that were highly reactive to Sm AWTA and not reactive to *Fasciola*

or *Echinococcus granulosus* were cloned by limiting dilution method, using splenocyte feeder layer according to Galfre and Milstein (1981). Isotypic analysis of MAb 12D/10F was done and proved to be of IgM class using a mouse hybridoma subtyping kit (Boehringer). Hybridoma cells were injected intraperitoneally into BALB/c mice for ascites production. Monoclonal antibody 12D/10F (IgM) was purified from ascitic fluid by euglobulin precipitation in distilled water according to Garcia-Gonzalez *et al.* (1988).

Characterization of target antigen recognized by monoclonal antibody

Target antigen recognized by monoclonal antibody 12D/10F was identified using the following techniques; immunoelectrophoresis (IEP) for determination of ability of MAb to recognize a repetitive epitope according to Capron *et al.* (1965). For identification of chemical nature of target antigen, the reactivity of MAb 12D/10F against the antigen before and after treatment with 20 mM sodium periodate and 4% trichloroacetic acid was tested for by indirect ELISA according to Woodward *et al.* (1985). For determination of molecular weight range of recognized antigen, polyacrylamide gel electrophoresis (PAGE) of antigen followed by enzyme linked immunoelectrotransfer blot technique (EITB) was performed according to Tsang *et al.* (1983).

Sandwich ELISA

Quantification of the target antigen of the MAb was achieved by sandwich ELISA using MAb 12D/10F both as antigen capture and detection antibody, being found to recognize an antigen with repetitive epitope. Labeling of MAb with horseradish peroxidase was performed by periodate method according to Nakane and Kawaoi (Nakane and Kawaoi, 1974).

After several optimization trials, the following sandwich ELISA originally described by Engvall and Perlmann (1971), was performed. Microtitration plates (Dynatech) were coated with 10 µg/ml of purified MAb in 0.1 M carbonate buffer, pH 9.6 dispensed as 100 µl/well and left overnight at room temperature. Plates were blocked by adding 200µl/well of 3% fetal calf serum/PBS/Tween for 1 hour at 37°C (3% FCS/PBS/T was used as diluting buffer and PBS/T as washing buffer). Undiluted sera were added (100 µl/well) and incubated for 2 hours at 37°C. Plates were washed with washing buffer. One hundred µl/well of 1:1000 dilution of peroxidase-conjugated MAb (5µg/ml) were added and incubated for 2 hours at room temperature, and then plates were washed as before. The reaction was

visualized by the addition of 100 μ l/well of O-phenylene diamine (OPD) substrate solution for 30 minutes in the dark at room temperature. The reaction was stopped by adding 50 μ l/well of 8 N H_2SO_4 and plates were read at 492 nm using ELISA microplate reader (Bio Rad).

Detection of circulating schistosomal antigen in serum by Latex agglutination test (LAT)

A polystyrene latex suspension (0.81 μ m; Sigma, St. Louis, MO) was used in this test. 1% standardized polystyrene latex suspension was prepared by mixing 0.1 ml of latex suspension with 9.9 ml of 0.02 M glycine-buffered saline (GBS), pH 8.4. This was stored at 4 °C until used. One ml of 1% latex suspension was mixed with 1 ml of purified MAb (1.0 mg/ml). The mixture was incubated at 37°C for two hours in a water bath. After incubation, antibody-sensitized latex particles were washed two times with GBS, pH 8.4, and centrifuged at 3000 x g for five minutes. The pellet of MAb-sensitized latex particles was emulsified with 1% bovine serum albumin/GBS, pH 8.4 to make a suspension of 2%. The particles were stored at 4°C until used. Latex particles coated with normal rabbit serum were used as negative control.

The test was performed on a clean slide divided with a glass marking pen into two halves. A drop of test serum or urine (50 μ l) was placed on each half of the slide. An equal volume of sensitized latex reagent was added to the serum or urine on one half. The same volume of control latex suspension was added to the serum or urine on the other half as a negative control. The slide was then manually rotated for two minutes then inspected. Agglutination with sensitized latex reagent and not with the control latex reagent was considered a positive result. Appropriate controls were examined in parallel in each test.

Interpretation of results: According to the intensity of agglutination accumulated around the edge of the reaction zone, the positivity was classified into high (+++), moderate (++) , low (+). When no agglutination was seen, the result was considered negative (-).

Statistical analysis

Data were expressed as mean \pm standard deviation (SD) or number (%). Correlations between different parameters were performed using Spearman's rank correlation coefficient. SPSS computer program (version 13 windows) was used for data analysis

3. Results

Characterization of target antigen recognized by monoclonal antibody

Immunoelectrophoresis of MAb versus AWTA in agarose gel showed a high density precipitation arc, proving the ability of MAb to recognize a repetitive epitope on AWTA (Fig. 1). The EITB technique revealed that MAb 12D/10F recognized *S. mansoni* AWTA antigen at 50, 60 and 65 kDa bands (Fig. 2). Binding of MAb 12D/10F to *S. mansoni* AWTA coated microtitration plates was strongly inhibited by treatment with 20 mM sodium periodate (39.0%) and slightly inhibited (9.0%) by treatment with 4% trichloroacetic acid denoting that target antigen for MAb 12D/10F was a proteoglycan.

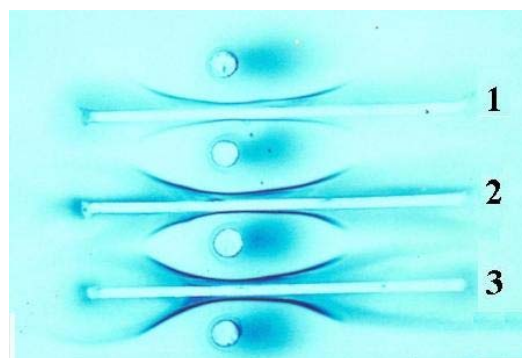


Fig. 1: Immunoelectrophoresis pattern of *S. mansoni* adult worm tegumental antigen (in wells) against 12D/10F MAb (in trough; 1: tissue culture supernatant, 2 and 3: ascitic fluid).

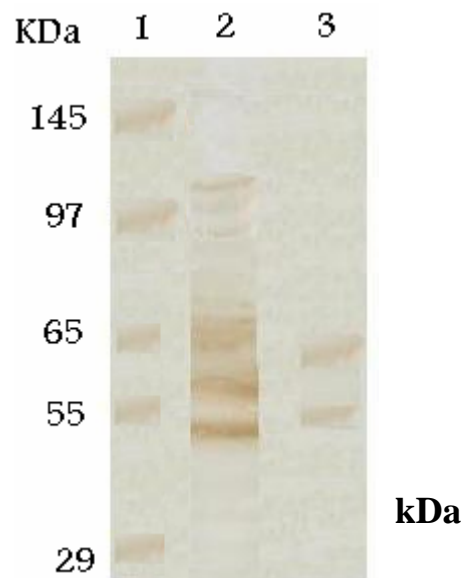


Fig. 2: Enzyme linked immunoelectrotransfer blotting pattern of *S. mansoni* tegumental worm antigen and *S. mansoni* SEA recognized by anti-*S. mansoni* AWTA MAb (12D/10F). Lane 1: Molecular weight standard proteins.

Lane 2: Pattern of *S. mansoni* adult worm tegumental antigen (AWTA).

Lane 3: Pattern of *S. mansoni* soluble egg antigen (SEA).

In urine, LAT was positive in 63 out of 70 *Schistosoma* infected patients and the sensitivity of the assay was 90%, while in serum LAT was positive in 61 out of 70 *Schistosoma* infected patients and the sensitivity of the assay was 87.1%. The seven and nine patients that showed false negative results in urine and serum respectively were among the light infection subgroup and the mean number of ova in their stool/g was 25.78 ± 9.31 and 28.49 ± 7.78 , respectively. The specificity of the assay was determined as the sum of results of negative control group and other parasites group. All the 30 negative controls were LAT negative in both urine and serum while 7 patients out of 32 other parasites group showed positive LAT in urine and 4 patients in serum and were considered as false positives. The specificity of the assay in urine and serum, therefore, was determined to be 88.7% and 93.5%, respectively. The diagnostic efficacy of the assay was 89.4% and 90.2%, respectively (Tables 1 & 2).

Levels of circulating schistosomal antigens in urine and serum of different studied groups were also measured by ELISA at OD readings equal to 492 nm. The cut off value was calculated as the mean OD

reading of negative controls + 2 standard deviation of the mean. The OD readings equal to or less than cut off value were considered negative while those readings greater than the cut off value were considered positive.

In urine, the cutoff was 0.245 and ELISA was positive in 65 out of 70 schistosomiasis patients and the sensitivity of the assay was 92.9% while in serum the cutoff was 0.390 and ELISA was positive in 67 out of 70 schistosomiasis patients and the sensitivity of the assay was 95.7%. Also, the 5 and 3 patients that showed false negative results in urine and serum were among the light infection subgroup. All the 30 negative controls were ELISA negative while 8 and 4 patients in other parasites group showed positive ELISA in urine and sera respectively and were considered as false positives. The specificity of the assay was 87.1% and 93.6%, respectively. The diagnostic efficacy of the assay was 90.2% and 94.7%, respectively (Tables 1 & 2).

In *S. mansoni* infected group, there was a significant positive correlation between schistosome egg count in stool and both the intensity of LAT and OD reading of ELISA in urine ($r = 0.847$; $p < 0.001$ and $r = 0.915$; $p < 0.001$, respectively) and in serum ($r = 0.863$; $p < 0.001$ and $r = 0.801$; $p < 0.001$, respectively) (Figs. 1 & 2).

Table 1: Mean ELISA OD readings at 492 nm in urine and serum samples classified according to intensity of LAT positivity in schistosomal group (n= 70).

Intensity of LAT	Urine		Serum	
	Positivity [n (%)]	ELISA (cutoff= 0.245)	Positivity [n (%)]	ELISA (cutoff=0.390)
High (+++)	19 (27.1%)	1.25±0.22	20 (28.6%)	1.59± 0.29
Moderate (++)	33 (47.1%)	0.85±0.12	28 (40%)	0.97 ± 0.14
Low (+)	11(15.7%)	0.52±0.11	13 (18.6%)	0.76 ± 0.13
No agglut.	7 (10.1%)	0.211±0.09	9 (12.8%)	0.299 ± 0.07

Table 2: Sensitivity, specificity and diagnostic efficacy of LAT and ELISA.

	LAT		ELISA	
	Urine	Serum	Urine	Serum
Sensitivity	90%	87.1%	92.9%	95.7%
Specificity	88.7%	93.5%	87.1%	93.6%
Diagnostic efficacy	89.4%	90.2%	90.2%	94.7%

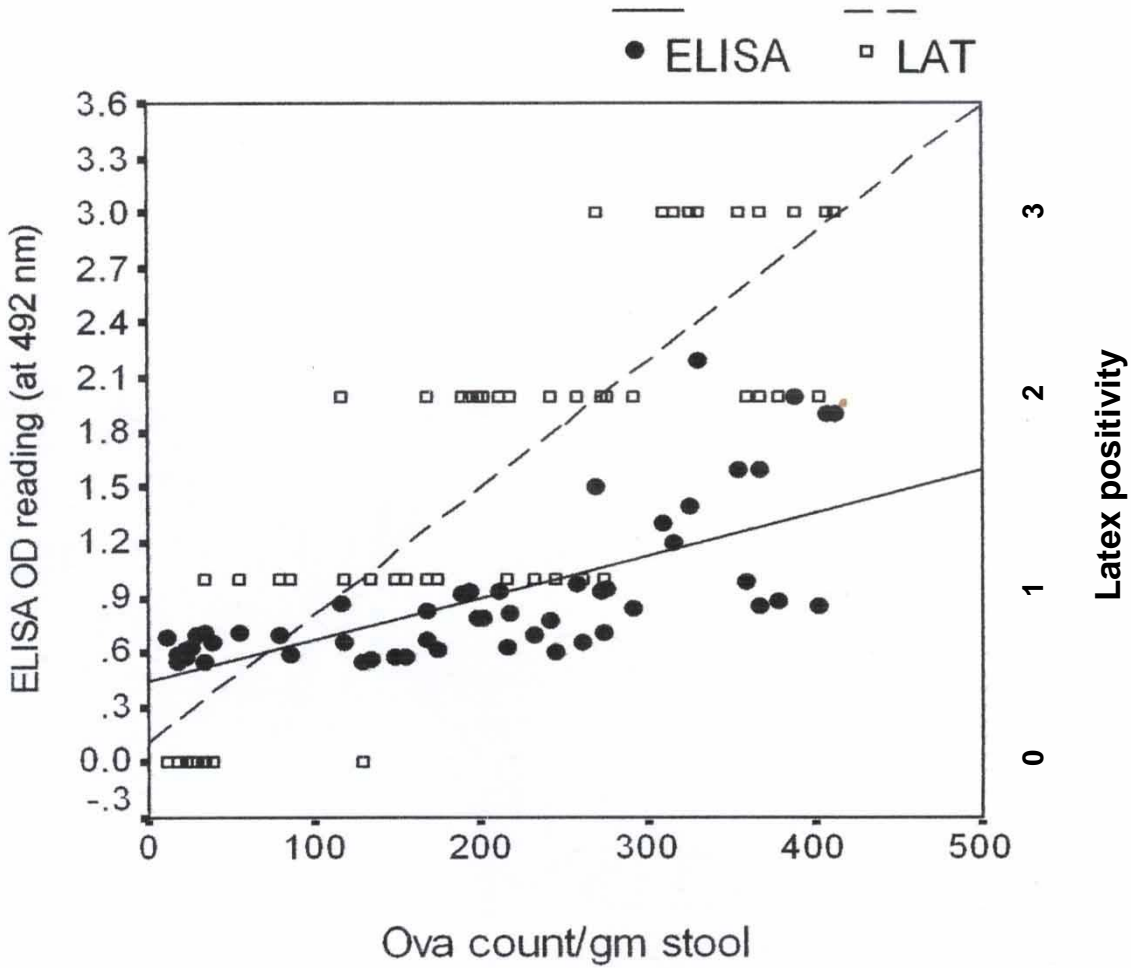


Fig. 3: Correlation between schistosome egg count in stool and both intensity of LAT positivity ($r=0.863$; $p< 0.001$) and OD reading of ELISA ($r= 0.801$; $p< 0.001$) in serum samples of *S. mansoni* infected group.

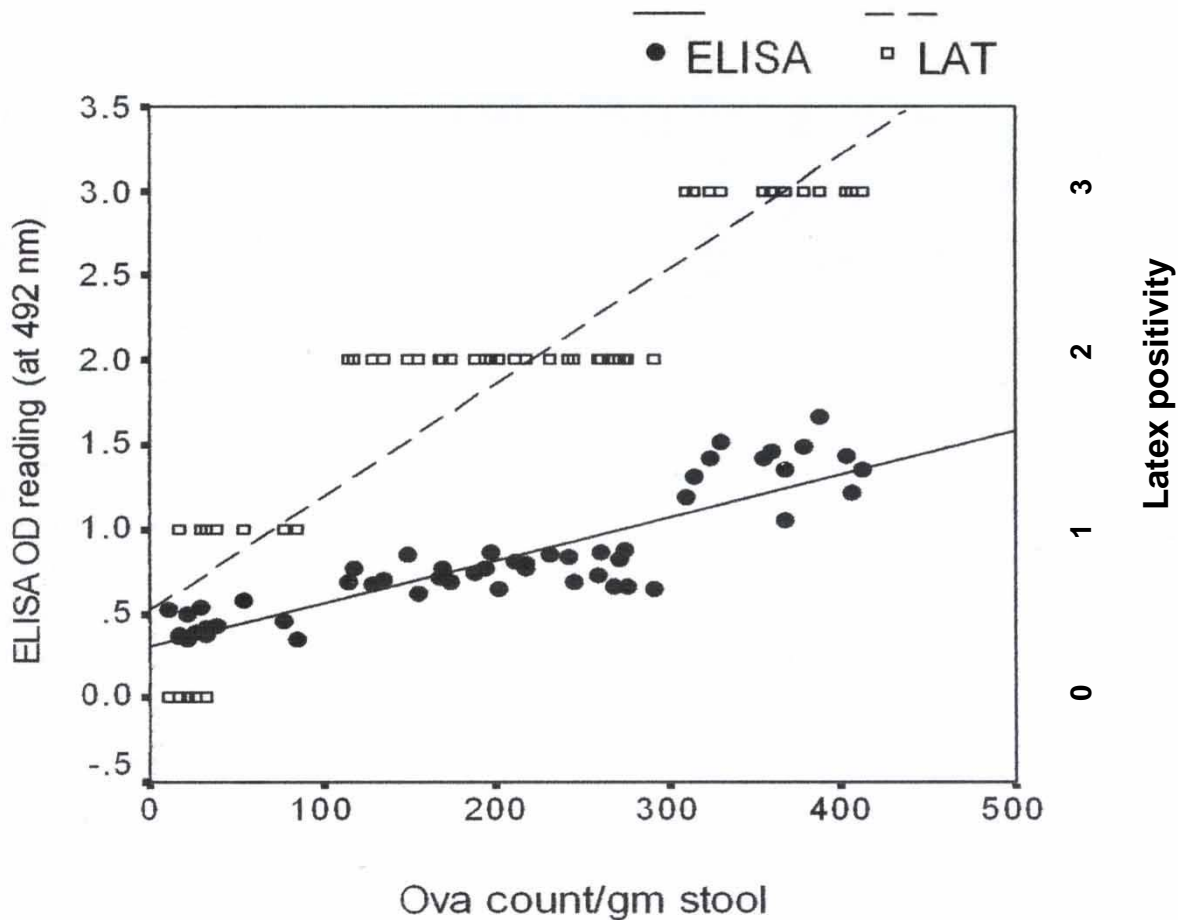


Fig. 4: Correlation between schistosome egg count in stool and both intensity of LAT positivity ($r= 0.847$; $p< 0.001$) and OD reading of ELISA ($r= 0.915$; $p< 0.001$) in urine samples of *S. mansoni* infected group

4. Discussions

The development of simple, rapid and sensitive methods for detecting CSA are needed as most available assays require several laboratory equipment and highly skilled persons.

Being able to recognize an antigen with repeating epitope on *S. mansoni* AWTA by IEP, MAb (12D/10F) was selected from a panel of MAbs and employed as both antigen capturing and detecting antibody in sandwich ELISA for detection of CSA in diagnostic extracts of *S. mansoni* and Pardo *et al.* (2004) denoted the 65 kDa as the major recognized band.

The latex agglutination test (LAT) is one of the simplest slide agglutination tests available in a diagnostic parasitology laboratory. The test has been used in the diagnosis of meningococcal meningitis (Gray and Fedorko, 1992). Since then, latex agglutination has been used to detect antibodies in a variety of parasitic diseases such as visceral leishmaniasis (Arya, 1997;

schistosomiasis patients' sera. MAb (12D/10F) is an IgM that recognized a proteoglycan antigen of *S. mansoni* AWTA in the molecular weight regions of 50-65 kDa. This is similar to anti-*S. mansoni* IgG MAb of Attallah *et al.* (1999b) which recognized a common band at 63 kDa in three stages of the parasite life cycle; cercariae, soluble egg antigen and adult worms. Moreover, using schistosomiasis patients' sera, Sulahian *et al.* (2005) detected three bands in the range of 65 to 120kDa as

Bagchi *et al.*, 1998), toxoplasmosis (Mazumder *et al.*, 1988), *Schistosoma japonicum* (Wang *et al.*, 2006) and *Echinococcus granulosus* (Barbieri *et al.*, 1993). Devi and Parija (2003) used LAT in detecting circulating *Echinococcus granulosus* antigens in serum, the sensitivity and specificity of the assay was 72% and 98%, respectively.

Although LAT has been used to detect antibodies to schistosomal antigens in serum, the test has

yet to be evaluated for the detection of CSA in urine and serum. The present study was carried out for detection of CSA in both urine and serum samples of a group of *S. mansoni* infected patients using a simple MAb based-latex agglutination test comparing its results with MAb-based sandwich ELISA as a well established reference test for CSA assay. A group of patients infected with parasites other than *Schistosoma* and healthy individuals group were also included in the study. The sensitivity of CSA assay in urine and serum samples by LAT was 90% and 87.1% respectively compared to 92.9% and 95.7% by sandwich ELISA. The specificity of LAT was 88.7% and 93.5% for CSA assay in urine and sera respectively versus 87.1% and 93.6% by sandwich ELISA. This means that sensitivity for CSA assay in urine samples by LAT was comparable to sandwich ELISA but specificity was higher. Therefore, the diagnostic efficacy for CSA assay in urine samples was slightly lower 89.4% using LAT compared to 90.2% by sandwich ELISA. However, sandwich ELISA gave a higher diagnostic efficacy for CSA assay in serum samples (94.7%) compared to 90.2% by LAT.

These results agree with those of Demerdash *et al.* (1995) and Hanallah *et al.* (1995) who used different MAbs-based sandwich ELISA for detection of CSA in both urine and serum samples of *S. mansoni* infected patients and reported a sensitivity of 90.0% and 94.8% in urine respectively, while in serum it was 97.0% and 98.4%, respectively. Also, El-Bassiouny *et al.* (2005) used a pair of MAbs in sandwich ELISA for detection CSA in serum samples of *S. mansoni* infected patients and found 96.7% sensitivity and 92% specificity.

Detection of CSA in urine has a potential for development of non-invasive screening test (Van Etten *et al.*, 1994; 1997; Polman *et al.*, 1995), while serum antigen detection may provide a more direct measure of worm burden for epidemiological research (Van Etten *et al.*, 1994; 1997; Polman *et al.*, 1995; Van Lieshout *et al.*, 1998).

In this study, a significant correlation was observed between the level of CSA detected by ELISA and LAT in both serum and urine and the number of eggs excreted in stool of schistosomiasis patients denoting the reliability of CSA detection as an indication for intensity of infection. These results were in parallel with those of Hendawy *et al.* (2006).

The false negative results observed in LAT and ELISA were found in patients with low number of ova/g stool and this could be due to the possibility that the intact ova of *S. mansoni* may release only small undetectable amounts of antigen into the circulation. Another possibility is that the antigen released from the parasite form immune complexes with circulating antibodies (Carlier *et al.*, 1983; Nash, 1984). Moreover, the disappearance of CSA could be due to the effect of successful chemotherapy denoting the

reliability of CSA assay as a cure monitor (Van Lieshout *et al.*, 1993; Demerdash *et al.*, 1995).

Finally, detection of CSA by LAT in urine is very simple, portable, non-invasive especially for children, sensitive and easy to perform a slide agglutination test. The test offers many advantages for its use in the diagnosis of *S. mansoni* infection in poorly equipped laboratories, does not require any special equipment or reagents, and can be performed under difficult field conditions. First and foremost, paramedical health personnel in a rural health center can perform this test on a microscopic glass slide. The test does not require any training or specific skills. Second, this test is cost-effective. Third, LAT is a rapid test, in which results can be obtained within minutes of performing the test. Finally, it can be used to monitor the efficacy of chemotherapy.

In conclusion, the use of LAT for CSA assay could be a valuable applicable screening diagnostic technique in field survey especially for urine samples. A confirmatory sandwich ELISA for CSA assessment in sera is recommended for query false negative results. At the same time, more studies have to be performed to improve the sensitivity and specificity of LAT and hence encourage its use on a large scale for diagnosis of multiple parasitic infections in field surveys.

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