

Novel Nanomagnetic Beads Based - Latex Agglutination Assay for Rapid Diagnosis of Human Schistosomiasis *Haematobium*

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Abstract—The objective of the present study was to evaluate the novel nanomagnetic beads based-latex agglutination assay (NMB-LAT) as a simple test for diagnosis of *S. haematobium* as well as standardize the novel nanomagnetic beads based –ELISA (NMB-ELISA). According to urine examination this study included 85 *S. haematobium* infected patients, 30 other parasites infected patients and 25 negative control samples. The sensitivity of novel NMB-LAT was 82.4% versus 96.5% and 88.2% for NMB-ELISA and currently used sandwich ELISA respectively. The specificity of NMB-LAT was 83.6% versus 96.3% and 87.3% for NMB-ELISA and currently used sandwich ELISA respectively. In conclusion, the novel NMB-ELISA is a valuable applicable diagnostic technique for diagnosis of human schistosomiasis *haematobium*. The novel NMB-ELISA assay is a suitable applicable diagnostic method in field survey especially when followed by ELISA as a confirmatory test in query false negative results. Trials are required to increase the sensitivity and specificity of NMB-ELISA assay.

Keywords—Diagnosis, Latex agglutination, Nanomagnetic beads, Sandwich ELISA.

I. INTRODUCTION

SCHISTOSOMIASIS is one of the most widespread parasitic infections of man and is second to malaria in socioeconomic and public health importance in tropical and subtropical areas. Schistosomiasis affects 230 million of the world's poorest people through 77 countries in tropical Asia, Africa, South America and Caribbean. However those at most risk of infection are in 52 countries [1], [2].

The diagnosis of schistosomiasis is traditionally achieved through the use of parasitological methods (urine filtration for *Schistosoma haematobium* and Kato-Katz thick smears for *S. mansoni* and *S. japonicum* infections). They are the most direct and specific way of detecting active infection, but often miss light-intensity infections [3]-[5], since only small amounts of excreta are examined. With preventive chemotherapy-based helminthiasis control programmes due to

be scaled up [6], [7], helminth egg output is likely to decline further and the problem of insensitive egg counting methods will be exacerbated [8], [9]. Considerable effort has been expended on the development of immunodiagnostic assays that will improve on microscopy. Circulating antigen detection assays are considered desirable since they are predicted most likely to reflect active infection. However, they have generally been shown to be no more sensitive than parasitological methods, particularly in situations where egg counts are low [10], [11].

Nanodiagnosics involve the use of nanotechnology in clinical diagnosis to meet the demands for increased sensitivity and early detection in less time. The large surface area of nanomaterials enables attachment of large number of target-specific molecules of interest for ultra-sensitive detection. Because of high sensitivity, nanotechnology enables detection of a few microorganisms or target molecular analytes specific to pathogens. Conventional methods are limited to achieve this ultra-sensitivity. In addition, unique properties of nanomaterials could allow rapid (as short as few minutes) and real-time detection of the pathogens. Moreover, relatively small sample volumes [12].

Nanoparticles (NPs) of various types have been primarily studied and have shown great promise for nanodiagnosics of infectious diseases. Nanoparticle technology based on fluorescent NPs (e.g. dye-loaded NPs, quantum dots (Qdots), magnetic NPs and metallic NPs (e.g. gold and silver NPs) has been successfully used to image, track and detect various infectious microorganisms [13]-[15]. Magnetic nanoparticles have been demonstrated to have exciting and promising applications in medical diagnostics and therapy [16], [17] as well as immunoassay based diagnostics [18]. In the immunoassay diagnostics, the functionalized magnetic particle bound to a bio molecule recognition unit is used as a label instead of enzymes or fluorescent material to capture the target analyte [19].

This work aimed to develop novel nanodiagnostic assays (nanomagnetic beads based- ELISA and nanomagnetic beads based- latex agglutination assay) for detection of circulating schistosomal antigen (CSA) in sera of human schistosomiasis *haematobium* and compared them with the traditional sandwich ELISA.

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II. MATERIAL AND METHODS

A. Studied Groups

This study was conducted on patients admitted to Tropical Medicine Department, Menoufiya University Hospitals and outpatients of Theodor Bilharz Research Institute (TBRI), Guiza. They were divided into three main groups. All patients and healthy volunteers were subjected to clinical and repeated parasitological stool examination using Kato-Katz technique [20], merthiolate iodine formaldehyde-concentration (MIFC) method and formol ether concentration techniques [21]. Urine analysis was performed for all studied groups using sedimentation method [22], nucleopore filtration method [23]. Ethical issues were strictly handled according to the International Ethical Guideline for Biomedical Research. Prior to urine, stool and blood collection the purpose of the study was explained to all individually participate in this study.

- Eighty-five *S. haematobium* infected patients were selected for this study, based on the presence of Schistosoma eggs in their urine.
- Thirty patients harboring other parasites than Schistosoma [Fasciola (n= 10), hydatid cyst (n=10) and Ascaris (n=10)].
- Twenty-five individuals of the medical staff at TBRI served as parasite free-healthy negative control.

All groups underwent detection of serum and urine circulating schistosomal antigen.

B. Processing of Samples

Urine samples: 1ml of freshly voided urine was boiled in water bath for 5 minutes and allowed to cool at room temperature before use.

Blood samples: sera were separated and stored at -70°C till required.

C. Preparation of Microsomal Fraction Antigen (PMA)

About 2000 adult worms of *S. haematobium* were disrupted by controlled homogenization in isotonic buffered at 4°C in 0.25 M sucrose solution (5 mM Tris-HCl pH 7.4) using three sequences of 10 passes of the pestle. Differential centrifugation of the homogenate yielded three particulate and one soluble fractions: the 480 x G pellet (nuclear), the 7650 x G pellet (mitochondrial), the 360,000 x G pellet (microsomal), and the 360,000 x G supernatant (cytosol). Quantitative analysis indicated a major concentration of specific antigenic activities in the microsomal fraction. Further purifications of the urea-solubilized, n-butanol-treated microsomal particles by gel filtration and ionic-exchange chromatography resulted in purified microsomal antigen (PMA) [24].

D. Characterization of PMA

The purified PMA was characterized for determination of molecular weight range using discontinuous Sodium Dodecyl Sulphate-Polyacrylamid gel electrophoresis (SDS-PAGE) in 12% slab gels (1mm thick), under reducing conditions (+2-mercaptoethanol) and stained with Coomassie blue (Bio-Rad) as described by Laemmli [25] and Takacs [26]. Followed by enzyme linked immunoelectrotransfer blot technique

(EITB) was performed according to Tsang et al. [27]. Apparatus and chemicals were purchased from Bio-Rad and both assemblies of apparatus and gel preparations were performed according to the operation manual.

E. Preparation of Polyclonal Antibodies (pAb) against PMA

Just before immunization, rabbit's sera were assayed by ELISA for *Shistosoma* antibodies and cross-reactivity with other parasites. Rabbits were injected intramuscularly (i.m.), with 1mg of PMA mixed 1:1 in complete Freund adjuvant (CFA) [28]. Then two booster doses were given, at 1 week intervals after the 1st injection each was 0.5mg antigen emulsified in equal vol. of incomplete Freund adjuvant (IFA) [29]. One week after the last booster dose, the rabbit's sera were obtained and pAb fraction was purified by 50% ammonium sulphate precipitation method [30]. More purification of pAb was performed by 7% caprylic acid method [31] and finally with gel-filtration [32]. The produced IgG appeared in a very high degree of purity except for few serum protein contaminants. Partially purified pAb was further adsorbed with fetal calf serum (FCS) to eliminate any non-specific binding with bovine antigen.

F. Sandwich ELISA

After several optimization trials, the following sandwich ELISA originally described by Engvall and Perlmann [33], was performed. Microtitration plates (Dynatech) were coated with 10µg/ml of purified pAb in 0.1 M carbonate buffer, pH 9.6 dispensed as 100µl/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 (100µl/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 pAb (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 µl/well of O-phenylene diamine (OPD) substrate solution for 30 minutes in the dark at room temperature. The reaction was stopped by adding 50 µl/well of 8 N H₂SO₄ and plates were read at 492 nm using ELISA microplate reader (Bio Rad).

G. Magnetic Microbeads Based- Sandwich ELISA

The microtitration plates were coated with 100µl/well of anti-*S. haematobium* antibodies coupled with magnetic microbead nanoparticle (5µg/ml) in carbonate buffer 0.06M, pH 9.6 and incubated overnight at room temperature. Plates were washed 3 times with 0.1M PBS/T, pH 7.4. The remaining sites in the wells were blocked by 200µl/well of 2.5% FCS/PBS/T and incubated for 2hr at 37°C. The plates were washed 3 times with PBS/T. 100µl of serum samples was pipetted into the wells in duplicate and incubated for 2hr at 37°C. The wells were then washed 3 times as before. 100µl/well of peroxidase-conjugated polyclonal antibodies of 1/3000 was then added and incubated for 1hr at room temperature. The reaction was visualized as above.

H. Magnetic Microbeads Based- Latex Agglutination Test (MMB-LAT)

A polystyrene latex suspension (0.81µm; Sigma, St. Louis, MO) was used in this test. 1% standardized polystyrene latex suspension was prepared by mixing 0.1ml of latex suspension with 9.9ml 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 of magnetic microbead nanoparticle was mixed with 1ml of purified pAb (1.0mg/ml). The mixture was incubated at 37°C for two hours in a water bath. After incubation, pAb -sensitized with magnetic microbead nanoparticle were washed two times with GBS, pH 8.4, and centrifuged at 3000 x g for five minutes. The pellet of pAb-sensitized magnetic microbead nanoparticle was emulsified with 1% bovine serum albumin/GBS, pH 8.4 to make a suspension of 2%. Latex particles coated with normal rabbit serum were used as negative control [34].

The test was performed on a clean slide divided with a glass marking pen into two halves. A drop of test serum or urine (50µl) was placed on each half of the slide. An equal volume of latex suspension sensitized magnetic microbead 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 (-).

I. Statistical Analysis

Data were expressed as mean ± 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.

Data were expressed as mean ± standard deviation; No.= number of individuals.

III. RESULTS

According to the number of ova count/10ml urine, the *S. haematobium* group was subdivided into light, moderate and heavy infection using nucleopore technique (Table I).

TABLE I
INTENSITY OF *S. HAEMATOBIMUM* INFECTION IN RELATION TO OVA COUNT

Subgroups	Infected human	
	Number	Ova count
Light (10-50)	22	25 ± 1.84
Moderate (60-100)	19	68 ± 3.92
Heavy (>100)	44	164 ± 8.31

A. SDS-PAGE and EITB

The SDS-PAGE analysis and Coomassie brilliant blue staining of PMA of *S. haematobium* are shown in Fig. 1. The fractionated *S. haematobium* PMA containing several polypeptide bands ranged from 220 to 17 KDa. Several bands were recognized when pooled sera from human infected *S. haematobium* were probed with fractionated PMA in immunoblot analysis (Fig. 1).

B. Reactivity of PMA by Indirect ELISA

The antigenicity of the PMA was characterized by indirect ELISA. Serum samples from *S. haematobium* infected patients gave a strong reaction against PMA with mean OD reading equal to 1.33 and no cross reactions were recorded with sample of patients infected with other parasites e.g., [*Fasciola* (n= 10), hydatid cyst (n=10) and *Ascaris* (n=10)].

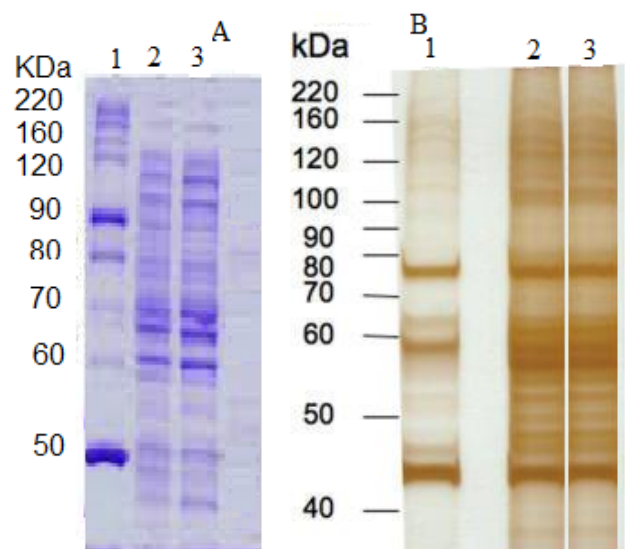


Fig. 1 (a) SDS-PAGE 1: Molecular standard weight 2. Crude microsomal antigen 3. Purified microsomal antigen and (b) EITB Molecular standard weight 2. Crude microsomal antigen 3. Purified microsomal antigen

TABLE II
REACTIVITY OF PMA BY INDIRECT ELISA

Parasitic antigen	OD readings at 492 nm
<i>S. haematobium</i>	1.33
<i>Fasciola</i>	0.261
hydatid cyst	0.233
<i>Ascaris</i>	0.149

C. Reactivity of Rabbit Anti- PMA pAb

Sandwich (S)-ELISA was performed to determine the reactivity of pAb which gave a strong positivity to against PMA till 1:3000 dilution. The optimization of various reagents was assayed by S-ELISA. The minimum PMA concentration used was 5ng/ml; the optimum concentration of purified IgG anti- PMA pAb was 10mg/dl, whereas conjugated IgG anti-PMA pAb was found to be 5mg/dl.

D. Detection of Circulating Ags in Human Sera by Sandwich ELIS

The levels of circulating schistosomal antigens in serum samples of different studied groups were measured by the purified IgG anti- PMA pAb-based sandwich ELISA. Ten out of 85 *S. haematobium* infected samples showed false negative results and the sensitivity of the assay was 88.2%, all false negative infected samples were belonged to the light infection patients where the mean ova count were $25 \pm 1.84/10$ ml urine. All the 25 negative controls were below the cut off value while 7 out of 30 of other parasites groups were at the border line of the cut off value giving 87.3 % specificity. Five false positive cases where belonged to the group of patient infected with *Fasciola* (Fig. 2).

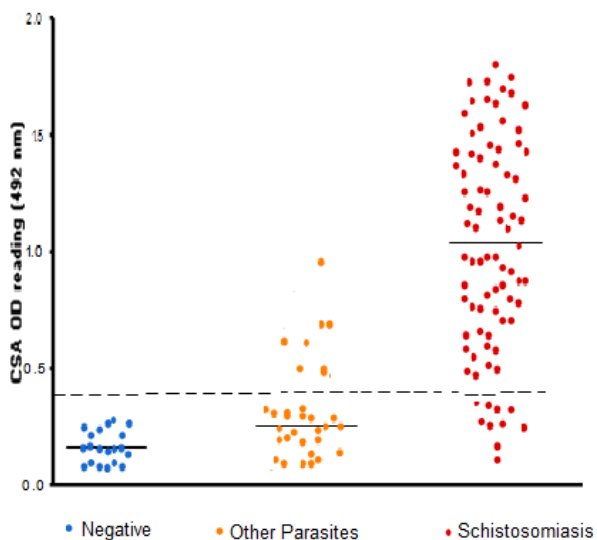


Fig. 2 Detection of circulating Ags in human sera by Sandwich ELISA

E. Detection of Circulating Ags in Human Sera by Magnetic Microbeads Based- Sandwich ELISA (MMB-S ELISA)

Three out of 85 *S. haematobium* infected samples showed false negative results and the sensitivity of the assay was 96.5%, all false negative infected samples were belonged to the light infection patients where the mean ova count were $25 \pm 1.84/10$ ml urine. All the 25 negative controls were below the cut off value while 2 out of 30 of other parasites groups were at the border line of the cut off value giving 96.3% specificity. The two false positive cases where belonged to the group of patient infected with *Fasciola* (Fig. 3).

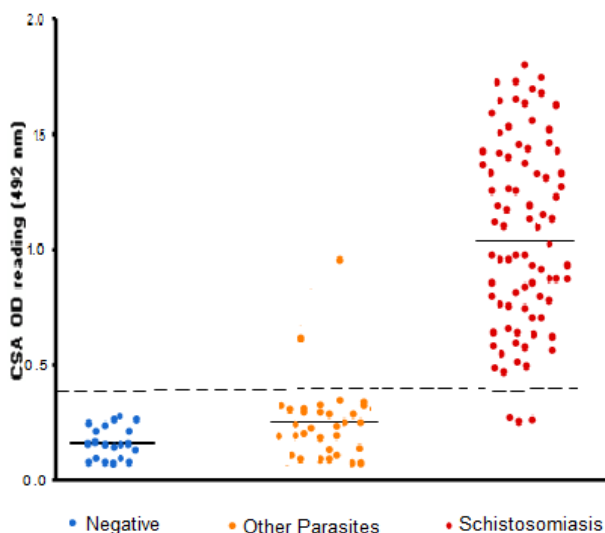


Fig. 3 Detection of circulating Ags in human sera by magnetic microbeads based-sandwich ELISA (MMB-S ELISA)

Fifteen out of 85 *S. haematobium* infected samples showed false negative results and the sensitivity of the assay was 82.4%, all false negative infected samples were belonged to the light infection patients where the mean ova count were $25 \pm 1.84/10$ ml urine. All the 25 negative controls were below the cut off value while 9 out of 30 of other parasites groups were at the border line of the cut off value giving 83.6% specificity. Seven false positive cases where belonged to the group of patient infected with *Fasciola* while the other two belonged to the group of patient infected with hydatid cyst (Fig. 4).

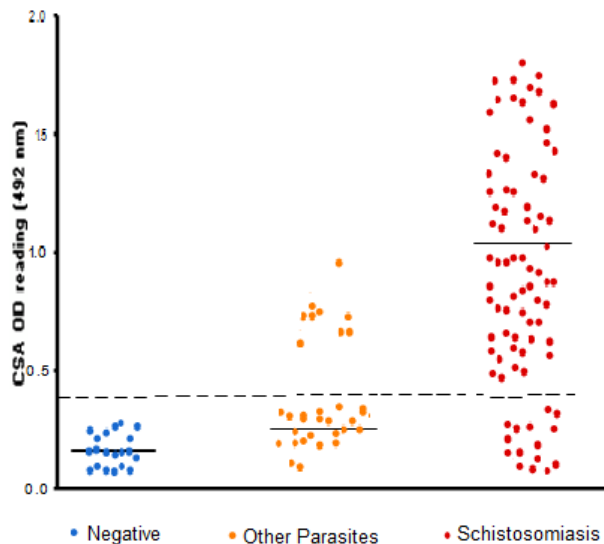


Fig. 4 Detection of circulating Ags in human sera by magnetic microbeads based- LAT

The following Table III summarizes the sensitivity and specificity, of sandwich ELISA, magnetic microbeads based-sandwich ELISA and magnetic microbeads based- LAT which

are used for detection of *S. haematobium* in human sera showing that the highest sensitivity and specificity were observed using magnetic microbeads based- sandwich ELISA -ELISA .

TABLE III
SUMMARIZES THE SENSITIVITY AND SPECIFICITY

	Sandwich ELISA	MMB-S ELISA	MMB- LAT
% sensitivity	88.2%	96.5%	82.4%
% specificity	87.3 %	96.3 %	83.6 %

Data were expressed as mean \pm standard deviation; No. = number of individuals.

IV. DISCUSSION

Schistosomiasis is one of the most important parasitic diseases in tropical areas. Approximately 200 million people worldwide currently suffer from the infection, which causes more than 500,000 deaths each year [35] with 20 million exhibiting severe symptoms [36]. Definitive diagnosis of urinary schistosomiasis is carried out by the detection of eggs in urine.

Nevertheless, the parasitological methods of diagnosis have low sensitivity in patients with the acute phase of the illness or with low- intensity infection [37]. In addition, day-to-day and circadian variation in egg excretion may lead to incorrect estimates in prevalence and intensity of infection [38]. To overcome this problem, several immunological tests have been developed for diagnosis of schistosomiasis [39]. Moustafa et al. [40] reported that antigen detection assays may facilitate earlier diagnosis than antibody tests, as production of detectable levels of specific immunoglobulin needs time.

In this study we have demonstrated that by using novel nanodiagnostic assay, using magnetic nanoparticles which can utilize larger surface area, a higher sensitivity can be achieved for detection of *Schistosoma* infections in serum samples as compared with Sandwich ELISA. MFA was used with complete and incomplete Freund's adjuvants for immunization of rabbits for preparation of anti-*Schistosoma* polyclonal antibodies. Adjuvants are usually used in immunization protocols in animals for many reasons. They can provide a depot for the immunogens at the site of injection allowing for slow, prolonged release of the immunogen in the animal and more important, they provide a mean of enhancing the immune response to the antigen. By using 12.5% SDS-PAGE technique under reducing condition, the PMF showed several immunogenic bands that was confirmed by EITB when pooled sera from human infected *S. haematobium* were probed with fractionated PMA in immunoblot analysis.

The reactivity of PAb was determined by indirect ELISA, gave a strong reactivity to *Schistosoma* PMF till dilution 1:3000. The optimization of various reagents was assayed by sandwich ELISA. The optimum concentration of purified IgG PAb was 10ug/ml, whereas conjugated IgG PAb was 5ug/ml. This yield of PAb was reasonable in comparison with the yield of purified immunoglobulin from any biological fluid

following similar purification procedures [41], [42]. The reactivity of the purified PAb was tested by indirect ELISA. It is of note that ELISA has been described as a valid test for detection of rabbit antibodies to fluke antigens, and has been the technique receiving most attention as an immunodiagnostic method for various parasitic infections [43]. Antigen detection assay in serum is generally performed by sandwich ELISA [44]-[48]. The magnetic microbeads based- sandwich ELISA -ELISA was comparable to that obtained with the sandwich-ELISA when the same samples were tested. Use of magnetic microbeads based offer the potential advantage of improving the sensitivity of the assay. The use of magnetic nanoparticles in immunoassay (nanomagnetic assay) combines the use of magnetic nanoparticles with a high binding capacity as a solid phase and the rapid reaction kinetics of solutions with the simple separation of bound and unbound materials on the solid phase, which provides the chance of enhancing the sensitivity of antigen detection [49], [50]. PMF detection in the serum samples of the tested groups by sandwich ELISA using magnetic nanoparticles, out of 85 Schistosomiasis cases 83 gave positive results, while 2 gave negative results, giving a sensitivity of 96.3%. On the other hand, PMA detection in the serum samples of the tested groups using sandwich ELISA only revealed a sensitivity of 88.2%.

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 [51]. Since then, latex agglutination has been used to detect antibodies in a variety of parasitic diseases such as visceral leishmaniasis [52], [53], toxoplasmosis [54], *Schistosoma japonicum* [55] and *Echinococcus granulosus* [56]. Devi and Parija [57] 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 serum samples of a group of *S. haematobium* infected patients using a simple magnetic microbeads based-latex agglutination test comparing its results with magnetic microbeads based- sandwich ELISA as a well-established reference test for CSA assay.

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