

Full Length Research Paper

Circulating antigens of *Schistosoma* parasites in urine of schistosomiasis patients in Central Sudan

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This study was aimed to detect circulating antigens of the schistosoma parasites in urine of schistosomiasis patients living in two camps, Hababna and Elshajara camps within Tyieba Elshiekh Elgorashi village, located North West from Hassahiesa city in the Gezira state- central Sudan. The prevalence of *S. haematobium* in the two camps was 15.4% and the prevalence of *S. mansoni* was 53.8%. Urine samples of individuals infected with *S. mansoni* (93), *S. haematobium* (13) and with both (co-infection, 19), were electrophoretically separated on SDS-PAGE and immunoblotted against confirmed schistosoma patient's serum. Immunogenic protein band with molecular weight 91 kDa was observed in all urine samples of individuals infected with *S. haematobium* and co-infection. In addition to 48 kDa in 8(61.5%), 11(57.9%) urine samples of infected individuals with *S. haematobium* and co-infection, respectively. A 60 kDa was found in 61(65.6%) urine samples of infected individuals with *S. mansoni*. No band was observed in urine samples of healthy control groups from endemic and non-endemic area. The present study showed that a 91 kDa protein is a highly immunogenic and may be a useful marker for diagnosing urinary schistosomiasis.

Key words: Circulating antigen, immunogenic protein, molecular weight, endemic area, urinary schistosomiasis.

INTRODUCTION

Schistosomiasis is the second major parasitic disease in the world after malaria, affects about 250 million people worldwide (Engels et al., 1996). The conventional ways of demonstrating parasite eggs in feces and urine for diagnosis of infection by schistosomes have drawbacks in sensitivity, especially in case of light infection and a relatively time consuming nature of the methods in application for epidemiological assessment and clinical use (Van Etten et al., 1994; Hamilton et al., 1998; Corachan, 2002; Van Dam et al., 2004). Several immunological tests have been developed for schistosomiasis diagnosis though only few resist scrutiny of effectiveness, reproducibility, cross reactivity and predictive values

(Rabello et al., 2002). The cumbersome procedures and the demand of sophisticated laboratory equipment and trained personnel make these few advanced approaches inappropriate and expensive for epidemiological applications in endemic areas. For instance, in seroepidemiological surveys, apart from test procedures, sample collection and processing are sometimes cumbersome and require trained personnel for venopuncture and separation of serum samples under field conditions. Besides that, venopuncture is not widely accepted, especially by children and certain ethnic and religious groups (Noya et al., 2002). For field-based diagnosis and follow-up of chemotherapy of schistosomiasis, there is urgent need for simple and reliable tests, which are applicable in endemic areas (Van Lieshout et al., 1992, 2000; Chitsulo et al., 2004). To answer the question of simplicity in immunodiagnosis of schistosome infection, some tests use urine sample in which the appropriate

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marker, either antibody or antigen, to be detected is expressed (Ren Li et al., 2004).

Immunological techniques which measure circulating anodic antigen (CAA), a negatively charged proteoglycan, and circulating cathodic antigen (CCA), a polysaccharide with a cathodic migration, have been developed and are being used for research purposes (Deelder et al., 1994). The existence of these circulating antigens in urine and serum without significant variation over short period of time (Disch et al., 1997; Polman et al., 1998) and rapid clearance after successful chemotherapy inspired some researchers to develop a simple and rapid dipstick as a good alternative to other conventional methods for the diagnosis of schistosome infection based on either genus or species specific monoclonal antibodies (De Jonge et al., 1990; Van't Wout et al., 1992; Deelder et al., 1994; Kremsner et al., 1994; Van Etten et al., 1994; Van Dam et al., 2004). Therefore, the present study was carried out to detect the circulating antigens of schistosome parasites in urine of schistosomiasis patients in central Sudan by electrophoresis separation and immunoblotting technique against schistosome patients' sera to know-how can these circulating antigens will contribute in the rapid, simple and reliable diagnosis and control of the disease.

MATERIALS AND METHODS

Study area and study population

This study was conducted in the two permanent agricultural camps in Gezira irrigated Scheme (Central Sudan). The two permanent camps (Hababna and Elshajara) located within Tayba Elsheikh Elgorashi village area, in the northern part of the scheme. The estimated population of each camp is about 500 individuals. The majority of the populations in the two camps are agricultural field labourers. Few individuals in the two camps are factory workers or government employees in Hassahiesa, the capital of the municipality in the northern part of Gezira State. The canal is the main source of water for the inhabitants of Hababna and Elshajara camps; although a low-yield well with a hand pump is exist in each camp.

Selection of study population and data collection

After explaining the aim of the study and obtaining informed consent from the leader of each camp and the inhabitants of the two camps, a total of 208 individuals 111(53.4%) male and 97(46.6%) female, age range from 4 - 80, years, were randomly selected out from the two camps. Then, a disposable small piece of clean plastic sheet and cup were given to each individual (guardian/parent in case of under 12 years children) to provide fresh stool and urine samples, respectively. Stool specimens were processed using developed modified Kato technique as previously described (Teesdale and Amin, 1976). Egg count was performed for *S. mansoni* and the intensity of infection was expressed as egg count per gram (EPG) of stool for each individual (WHO, 2002). Urine specimens were examined by Sedimentation technique (Braun-Munzinger, 1986).

Blood and urine samples were collected from infected individuals and healthy control from endemic and non-endemic area. Circulating antigens in urine samples were detected by electrophoresis separation and immunoblotting technique against schistosome patient's sera.

Urine samples preparation

Urine specimens were collected from infected individuals with *S. haematobium*, *S. mansoni*, and with both (co-infection) and healthy controls from endemic and non-endemic area in 15 ml tubes after parasitological examinations. Specimens were centrifuged at 3000 rpm for 10 min and then supernatant discarded and about 20 µl of the residue and the pellet of urine mixed well with 100 µl of the phosphate buffer saline (PBS) and stored at -20°C until used.

Serum samples

Two ml blood in non-heparinized tube was collected from infected individuals with schistosomiasis. Blood was allowed to clot at room temperature, and then sera were separated by centrifugation at 2000 rpm for 15 min and stored at -70°C till use.

Detection of schistosome circulating antigens

Separation and electrotransfer of proteins in urine sample:

Prepared urine samples after thawing and mixing well by vortex were separated in 15% reducing and discontinuous SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli, (1970) at 100v for 2 h by using slab Mini-Gel system (Mini-V 8 - 10 Vertical Gel Electrophoresis Apparatus, BRL, Life Technologies Inc., Gaithersburg, USA) to separate the proteins in urine samples according to their molecular weights and then separated proteins were transferred to a nitrocellulose paper (NCP) according to Towbin et al. (1979) method using a minigel transfer unit. The transfer was performed at a constant voltage of 150 v for 1 h.

Immunoblotting (Western Blotting): An Enzyme immunoassay (EIA) was used to detect the immunogenic protein bands on the nitrocellulose paper. Each nitrocellulose paper (NCP) was blocked by incubated in 0.3% PBS-Tween 20 (T20) at room temperature with continuous shaking for 1 hour. After three times wash in 0.05% PBS/T20 the membranes were incubated with confirmed schistosome patients' sera (1:50 dilutions) in 3% Bovine Serum Albumin (BSA) at room temperature for 1 h with continuous shaking. The membranes were then washed three times in 0.05% PBS/T20 to remove unbound antibodies. The anti-human IgG conjugated with horseradish peroxidase enzyme (Sigma- Aldrich Co.) was added at a dilution of 1:2000 in 0.05% PBS/T20 to the NCP and incubated at room temperature for 1 h with continuous shaking. Then washed in 0.05% PBS/T20 as before and soaked in 3, 3'-diaminobenzidine (DAB) substrate solution, on continuous shaking until clear bands developed. The reaction was stopped by washing the NCP in distilled water. The developed membranes were left to dry and photographed using digital camera and molecular weights of developed bands were calculated.

Molecular weight calculations: The immunogenic protein bands which developed in the nitrocellulose membranes were analyzed by computerized programme (UN-SCAN-IT gel Demo software) downloaded from the website <http://www.silksscientific.com/usidemo.htm> to calculate the molecular weight of the protein bands in contrast with molecular weight of standard marker (Sigma-SDS-6H).

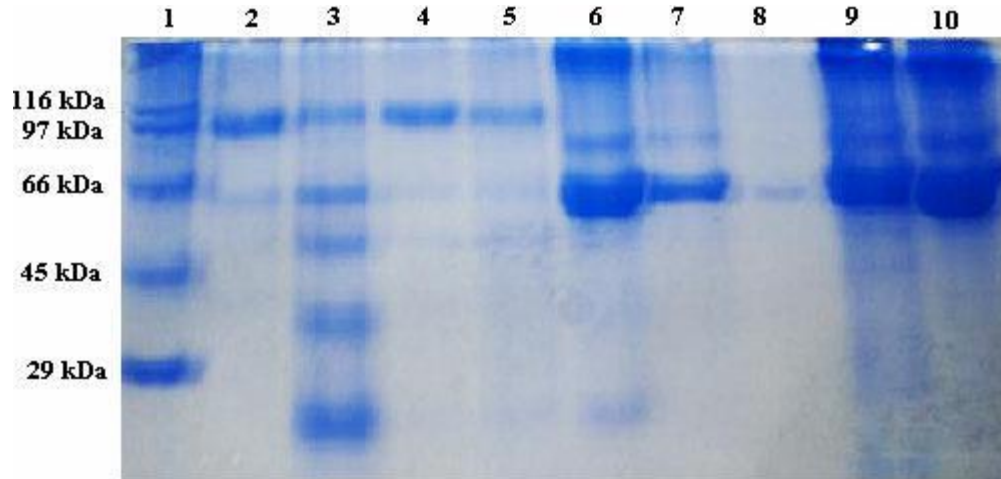


Figure 1. SDS-PAGE separations of urine samples stained with comassie brilliant blue. Lane 1: Molecular weight marker, Lane 2: Healthy control from non endemic area, Lane 3: Healthy control from endemic area, Lanes 4 and 5: *S. mansoni* infected individuals, Lanes 6 and 7: co-infection infected individuals, Lane 8: Empty (no sample), Lanes 9 and 10: *S. haematobium* infected individuals.

RESULTS

Prevalence and intensity of schistosoma infections

The prevalence of *S. haematobium* in the two camps was 15.4% and the intensity of infection was 0.9 Geometric mean egg count (GMEC). Whereas the prevalence of *S. mansoni* was 53.8% and the intensity of infection was 2.04 (GMEC) according to parasitological examinations by Kato and sedimentation techniques.

Separation of urine samples

Electrophoretic separation of urine samples were appeared various protein bands with different molecular weights when were stained with comassie brilliant blue stain as shown in Figure 1. Molecular weights of the protein bands were ranged between 11 - 102 kDa. Immunogenicity of these protein bands were tested by the immunoblotting (Western blot) technique.

Immunoblotting (Western blotting)

Electrophoretically separated proteins from urine samples were transferred to NCP and immunoblotted against the schistosoma patients sera revealed an immunogenic protein bands with apparent molecular weight 91 kDa in all urine samples of individuals infected with *S. haematobium* and co-infection, in addition to 48 kDa band in 8(61.5%), 11(57.9%) of urine samples of infected individuals with *S. haematobium* and co-infection, respectively. While a single 60 kDa band was detected in 61(65.6%) of urine samples of infected individuals with *S. mansoni*.

Whereas no band was observed in urine of healthy control groups from endemic and non-endemic area. The soluble worm antigen of *S. mansoni* (SWA) was used as a positive control (Figure 2).

DISCUSSION

Diagnosis of human schistosomiasis is very central to make a decision on individual case management, at all stages of control programs and for comparing control programs (Feldmeier et al., 1993; Sturrock, 2001). The conventional ways of demonstrating parasite eggs in feces and urine for diagnosis of infection by schistosomes have drawbacks in sensitivity, especially in case of light infection and a relatively time consuming nature of the methods in application for epidemiological assessment and clinical use (Van Etten et al., 1994; Hamilton et al., 1998; Corachan, 2002; Van Dam et al., 2004). These drawbacks attracted researchers' attention for innovation of new approaches which are reliable, easy to perform and are not much demanding in terms of equipment as well as trained manpower (Van Etten et al., 1994; Van Dam et al., 2004). As we know the occurrence and development of schistosomiasis is strongly depended on human immunity against schistosomes (Mountford et al., 1994; Yokoi et al., 1996). Thus, analysis of schistosome antigens is useful not only for understanding of immunoprophylaxis and of the immunological pathogenesis of this disease but also for providing antigens for establishing a specific, sensitive diagnostic technique (Mountford et al., 1994; Yokoi et al., 1996). The excretion of the schistosome-circulating antigens in the urine of infected patients makes possible the use of noninvasive techniques for simple diagnosis of schistosomiasis

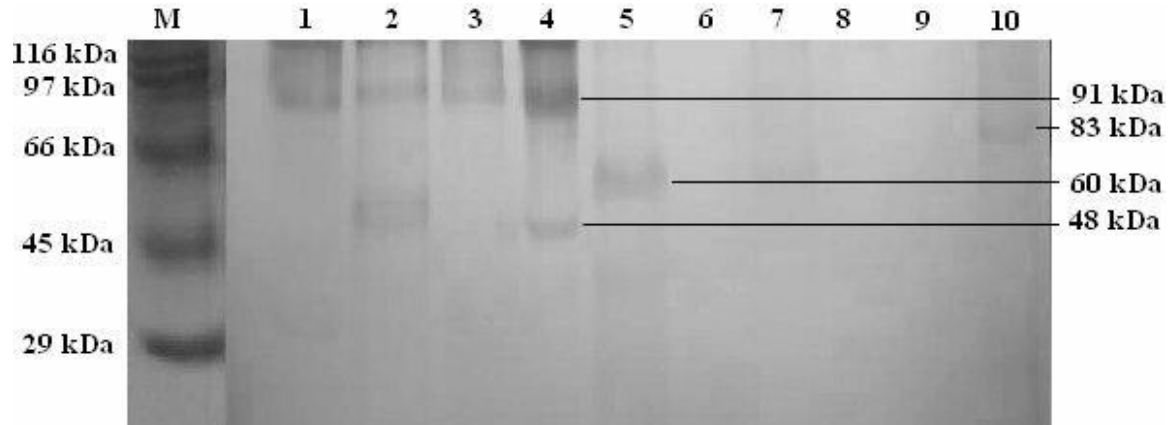


Figure 2. Western blot of separated urine samples. M: Molecular weight marker, Lanes 1 and 2: *S. haematobium* infected individuals, Lanes 3 and 4: Individuals infected with *S. mansoni* and *S. haematobium* (co-infection), Lanes 5 and 6: *S. mansoni* infected individuals, Lane 7: Healthy control from non-endemic area, Lane 8: Healthy control from endemic area, Lane 9: Empty, Lane 10: SWA of *S. mansoni*.

(Feldmeier,1993; Deelder et al., 1996). In various previous studies, sensitivities for circulating antigens in general have been reported to be lying between 65 and 85% (De Jonge et al., 1991; Van Lieshout et al., 1992; Stothard et al., 2006). Similar results were obtained in a study carried out by Legesse and Erko (2007) in schistosomiasis mansoni endemic focus in Ethiopia. Furthermore, urine-CCA has been claimed to be useful in the diagnosis of schistosomiasis while a recent study by Stothard et al. (2006) revealed its absolute failure in detecting *S. haematobium* antigen. In studies done in Zanzibar, Niger and Burkina Faso, the CCA strip was not able at all to detect active cases of urinary schistosomiasis while it showed promising results in *S. mansoni* infection (Ayele et al., 2008). Despite the many advantages of currently used reagent strip test like field applicability, ease in collecting many urine samples and testing within a short time which facilitate the field activities, many results of previous studies revealed a relatively reduced sensitivity of reagent strip test. Since sensitivity of a test is an important requirement that truly shows a diseased subject, the relative insensitivity of the reagent strip test necessitates for the improvement of its sensitivity (Legesse and Erko, 2007, 2008; Ayele et al., 2008). Generally previous studies were corroborated on the importance and significance of circulating antigens as successful and promising alternative diagnostic method for schistosomes infection but further study is needed to improve its sensitivity and specificity. Therefore, the present study was aimed to detect the circulating antigens of schistosome parasites in urine of schistosomiasis patients by using immunoblotting technique (Western blot) which could be have a high sensitivity and then suitable for serodiagnosis. Western blot experiments revealed the presence of immunogenic protein band with molecular weight 91 kDa in all urine samples of individuals infected with *S. haematobium* and co-infection. In

addition to 48 kDa in 8(61.5%), 11(57.9%) urine samples of infected individuals with *S. haematobium* and co-infection, respectively. A 60 kDa was found in 61(65.6%) urine samples of infected individuals with *S. mansoni*. Appearance of 48 kDa and 60 kDa circulating antigens in some urine samples of infected individual's and disappeared in other may be due to the liberation of these antigens in a little amount by schistosome parasite, so the preparation urine sample method which used in the present study may need some modification to obtain better results. The presence of a 74 kDa circulating antigen in the urine of *S. mansoni* infected individuals was examined using Fast Dot-ELISA based on MAb BRL4 by Attallah et al. (1998). Also detecting the 63 kDa circulating antigen in urine samples of infected patients with *S. mansoni* by using the Fast Dot-ELISA as a diagnostic tool was examined by MAb C5C4 (Attallah et al., 1999).

It is of interest that an antigen with a similar size has not been previously reported, unless 63 kDa band which detected in urine of *S. mansoni* patients by Attallah et al., (1999) seem to be close to 60 kDa reported in this study. This study could be more comprehensive, because it was involved *S. mansoni*, *S. haematobium* and even the co-infection to give more information about the circulating antigens of each species. The appearance of a 91 kDa circulating antigen in the present study in all urine samples of infected individuals with *S. haematobium* and co-infection suggested that this antigen attributed to *S. haematobium*, so the present study suggested that 91 kDa protein is a highly immunogenic and may be a useful marker for diagnosing urinary schistosomiasis.

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