

Identification of A 38 KDa Antigen in the Urine of Schistosoma Mansoni Chronically Infected Patients Using A Specific Antibody Microeluted from A Nitrocellulose Membrane; A Diagnostic Tool

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ABSTRACT

Schistosomiasis is a chronic debilitating disease affecting 200 -300 million throughout the world, a major focus of research has been done to identify and characterize antigens that may have vaccine and/or diagnostic potential. In the present study, NP-40 extracted surface proteins of Schistosoma mansoni (S. mansoni) adult worms were subjected to 12.5% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and electrotransferred onto a nitrocellulose membrane (PVDF), then, incubated with pooled sera collected from S. mansoni chronically infected patients. The presence of antibodies identify a number of NP-40 extracted surface proteins of the adult worms of S. mansoni. One of these proteins of 38 kDa molecular weight with high immunogenicity was selected. The strip of nitrocellulose membrane containing the complex of the identified protein and its specific antibody was cut guided by the molecular weight marker, then, the antibody was micro-eluted. Proteins of urine samples from the same patients were precipitated and purified over G-Sephadex column. The purified proteins of urine samples and proteins of sera were subjected to another SDS- PAGE and electro-transferred onto PVDF membrane. The microeluted antibody was used to identify an antigen of 38 kDa molecular weight in sera of the chronically infected patients as well as in urine. The 38 kDa antigen was excreted in the urine of those patients in a stable form and detected by the specific monoclonal antibody. The active epitope of 38 kDa antigen could be a promising immunochemical probe for S. mansoni infection diagnosis. Further studies will be done to characterize that antigen as well as its potential application in immunodiagnosis for S. mansoni.

INTRODUCTION

Bilharzia (schistosomiasis) occurs in the tropics and subtropics and is one of the most important parasite diseases of humans¹.

Satisfactory diagnostic techniques are prerequisite for the success of schistosomiasis control programs in endemic areas. Parasitological

methods lack sensitivity, so, serological tests have been suggested to be incorporated for schistosomiasis diagnosis. Schistosomiasis diagnosis by detection of specific antibodies is likely to be more sensitive than the traditional method of diagnosis by detection of eggs in stool or urine².

The purification, identification and characterization of schistosome

antigens are needed as a step toward the diagnosis of human schistosomiasis and the development of a vaccine against the parasite.

The improvement of the immunodiagnostic tools depends upon the production of specific and sensitive antigens in sufficient amount to provide low cost assays. Some recombinant proteins and synthetic peptides as well as the circulating antigens of *S. mansoni* released from the surface of the adult worms as secretory-excretory or gut associated antigens are released directly into the host blood stream³, might be used as immunogenic and as specific antigens for diagnostic purposes in schistosomiasis⁴.

Several investigators had identified and characterized many of these antigens in the urine of *S. mansoni* infected patients using monoclonal antibodies that have a potential application in immunodiagnosis^{5,6}.

⁷reported the potential value of synthetic peptides for the development of diagnostic tests based on antibody mediated capture of stable circulating antigens and confirmed the strategy of using enzyme antigens for diagnosis.

The aim of the current study was the identification of a secretory-excretory protein fraction of *S. mansoni* adult worms with high immunogenicity in the urine of chronically infected patients with *S. mansoni* using a specific monoclonal antibody microeluted from PVDF membrane against one of the adult worms surface proteins with high immunogenic potential to be used as a biomarker for *S. mansoni* diagnosis.

MATERIALS & METHODS

The preparation of Nonidet P-40 (NP-40) soluble surface proteins extract of the adult worms of *S. mansoni*⁸. Adult worms were mixed with NP-40 buffer containing 20 mM Tris, pH 7.4, 0.1% NP-40 and 2 mM phenyl methyl sulfonyl fluoride (PMSF), then, vortexed every 5 min for 1 hr. The pellet was spun down and the supernatant was collected and stored at -20°C.

Sera and urine samples were obtained from chronically infected patients with *S. mansoni* from Tropical Medicine Outpatient Clinic, Zagazig University Hospitals.

Preparation of proteins of urine⁹. Proteins are precipitated with fully saturated solution of ammonium sulfate, then, purified using G-Sephadex column. The elute from column was dialyzed against 2 liters of Phosphate-Buffered Saline (PBS) (0.4 g NaCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄/liter).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)¹⁰. The resolving gel (12%) and stacking gel (5%), the gel was left 30 min for polymerization, each one of the sera and urine samples (10 µl each) was mixed with an equal volume of 2 X SDS(sodium dodecyl sulfate) gel loading buffer {1.25 ml 1 M Tris, pH 6.8, 4.0 ml 10% SDS, 2.0 ml glycerol, 20 mg bromophenol blue, 1.0 ml β-mercaptoethanol and deionized distilled water (dd H₂O) up to 10 ml}, the samples were boiled for 5 min, then, loaded onto the gel. Electrophoresis was carried with constant current of 20–25 mAmp until the dye arrived at the gel front. The

gel was stained in Commassie stain with rocking for 1-2 hrs, then, destained till acceptable back ground was obtained and left to dry.

Western blot analysis¹¹. After electrophoresis without staining, the gel was placed in a sandwich form with a piece of PVDF membrane in a western transfer buffer (3.02 g Tris-base, 18.8 g glycine, 200 ml ethanol and dd H₂O up to a liter), the transfer was carried out at 75-100 volts for 1 hr. The membrane was blocked in 5% non fat dry milk in PBS, washed 4 times in PBST(0.05% Tween-20 in PBS), then, incubated with the microeluted antibody (the primary antibody) for 3 hrs at room temperature (RT). The secondary antibody (Anti-rabbit IgG conjugated with alkaline phosphatase) was incubated with the membrane for 1 hr at RT, then, washed 4 times in PBST. The substrate color reagent (Vector Laboratories) was dissolved in 0.1 M Tris- HCl, pH 9.5 and incubated with the membrane until good signals were developed, then, left to dry.

Microelution of antibodies from PVDF strips¹². The strip of PVDF membrane that contains the band of the selected protein after transfer was cut guided by the molecular weight marker. The strip was washed 3 times in TN buffer (10 mM Tris- HCl, pH 8.0, 0.15 M NaCl/liter) 20 min each, then, in 0.1 M boric acid and lastly in

PBS. For elution of antibody, the membrane was incubated in 0.1 M glycine, 0.15 M NaCl, pH 2.6 for 2 min with gentle rocking, then, its pH was adjusted to pH 8.0 by 2 M Tris-HCl, pH 8.0 and frozen at -70°C.

RESULTS

Detection of *S. mansoni* high immunogenic surface proteins using sera obtained from *S. mansoni* chronically infected patients. Sera containing antibodies were used to identify some antigenic epitopes of the surface proteins of *S. mansoni* adult worms by SDS-PAGE and western blotting techniques. The NP-40 extracted surface proteins of *S. mansoni* adult worms were separated by SDS-PAGE, then, subjected to electro-transfer onto PVDF membrane which was probed with chronically infected patients with *S. mansoni* sera's after blocking the non-specific binding sites with 5% non fat dry milk in PBS. Sera's antibodies identified antigens with different molecular weights, some of them showed high immunogenicity. One of the identified antigens having 38 kDa molecular weight was selected to be studied. The molecular weight marker was indicated on the side of PVDF membrane (**Fig .1**).

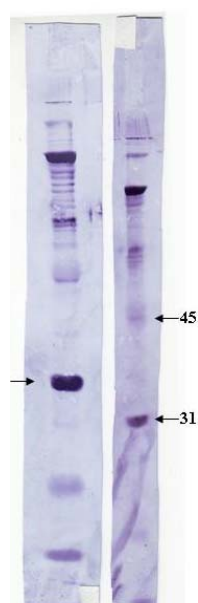


Fig. (1) Immunoblot of NP-40 extracted surface proteins of *S. mansoni* adult worms probed with *S. mansoni* chronically infected patients sera's. The selected protein having 38kDa molecular weight was pointed at by an arrow.

Detection of *S. mansoni* antigen in the urine of *S. mansoni* chronically infected patients. The antigenic epitope reactive with the specific monoclonal antibody microeluted from PVDF membrane was identified in the urine samples obtained from *S. mansoni* chronically infected patients by 12.5% SDS-PAGE and western blot techniques, samples of urine from normal persons not infected with *S. mansoni* were used as negative control. The microeluted antibody identified a protein with an apparent molecular weight of 38 kDa in the

urine samples of patients as well as the same patients sera's (as positive control). The antibody did not react with proteins of sera and urine samples from the non-infected individuals as well as proteins of *E. coli* lysate (used as negative control). (**Fig. 2**).

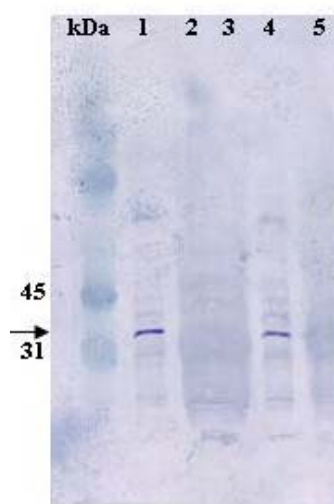


Fig. (2). Immunoblot of sera's proteins of *S. mansoni* chronically infected patients in lane 1, the precipitated and purified proteins of urine samples of the same patients in lane 4, while, lanes 2 and 3 are sera's proteins and the precipitated and purified proteins of urine of the non infected individuals with *S. mansoni* as positive control, *E. Coli* lysate is in lane 5 as a negative control. The molecular weight marker is indicated on the left side of PVDF membrane and the arrow points at the identified protein (38 kDa) by the microeluted monoclonal antibody.

DISCUSSION

Schistosomiasis is a global health problem caused by several species of schistosome blood flukes. It is endemic in 74 developing countries, 200 million people are infected worldwide¹³, causing an estimated 200,000 deaths / year¹⁴. Thus schistosomiasis has earned a category II disease ranking next to malaria for importance as a target tropical disease by World Health Organization Special Programme for Research and Training in Tropical Disease¹⁵.

Diagnosis of *S. mansoni* is confirmed by microscopic detection of eggs in stool or organ biopsies. The sensitivity of these procedures is variable due to fluctuation of egg shedding. The development of novel methods for parasitological diagnosis that are both highly sensitive and low in cost has been strongly recommended by the World Health Organization¹⁶.

Alternative approaches based on antibody, antigen and DNA detection are of potential application¹⁷.

Diagnosis of schistosomiasis by detection of specific antibodies is likely to be more sensitive than diagnosis by traditional parasitological techniques, where circulating antigen assays can be used not only for diagnosis but also, for estimating intensity of infection¹⁸.

Many antibody techniques have been developed for *S. mansoni* diagnosis as indirect immunofluorescence, enzyme linked-immunosorbent assay (ELISA) and indirect hemagglutination.

¹⁹suggested that people who are parasitologically negative and /or circulating antigen negative can not be assumed non infected as well as positive serological test results do not necessarily prove an infection due to imperfect techniques as cross reactions may result in false positive results. The immunological methods such as ELISA required more advanced laboratory setting but may yield a higher sensitivity particularly for antibody detection but treatment efficacy remains difficult to determine since specific antibodies continue to present long after the worms have disappeared. Detection of parasite antigens as circulating cathodic and anodic antigens (CCA and CAA) by ELISA shows many advantages, such as demonstration of active infections or treatment effect and has a high specificity²⁰.

The sensitivity, specificity, positive and negative predictive values of a reagent strip test for the diagnosis of *S. mansoni* by detecting circulating cathodic antigen (CCA) in urine were evaluated using 184 stool and urine samples collected from school children living in relatively low endemic area of schistosomiasis mansoni living in Ethiopia, the results were slightly higher than the stool techniques (65.2 vs 42.4, $p > 0.05$) and the sensitivity was low in case of light to moderate infections²¹.

Immunodiagnostic methods based on detection of specific antibodies continue to be the most effective and practical methods for diagnosis of schistosomiasis and correlated clinically with intensity of infection morbidity and post-treatment

monitoring²², their sensitivities lie between 65 and 95% according to different studies^{23,24}.

Few serological tests have high sensitivity and excellent specificity for *Schistosoma* diagnosis²⁵. Western blot analysis is an interesting tool for the detection and confirmation of schistosomiasis, the ease of use, good sensitivity and specificity makes western blot useful test for diagnosis of the disease²⁶. Some investigators reported the usefulness of western blot analysis for differentiating between recent and chronic *Schistosoma* infection²⁷ and different *Schistosoma* species.

The technique of Western blot could be used worldwide for analysis made with crude *S. mansoni* antigens which is the most widely used for diagnosis of schistosomiasis²⁸. The fundamental advantage of using complex antigens from the adult worms is due to their high yield of antigenic material².

The development of monoclonal antibody had led to characterization of several diagnostic and protective antigens. An antigen of 28 kDa was purified by²⁹.

³⁰ had purified 45 kDa and 30 kDa glycoprotein antigens from *S. mansoni* soluble cercarial extract.

⁵ identified a circulating cathodic antigen at 41/42 kDa in the urine of *S. mansoni* infected patients using monoclonal antibody. ³¹ identified a 74 kDa antigen in three developmental stages of *S. mansoni* and in the urine of *S. mansoni* infected patients using anti-*S. mansoni* monoclonal antibody.

The detection of specific antigens allow species diagnosis, 30

kDa band detection is highly specific for *S. mansoni* infection and the detection of 23 kDa band is specific for *S. hematobium* infection²⁸. In an attempt to develop more sensitive diagnostic tools for schistosomiasis detection, in the present study, the microeluted specific antibody was used to recognize a single 38 kDa antigen excreted in the urine of *S. mansoni* chronically infected patients as determined by western blotting, so, the identified antigen can be used as a reagent for immunodiagnosis of *S. mansoni*.

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REFERENCES

1. **Dominic, W., Marcus, P., Thomas, Q., Stefanie, K., Gerd-Dieter, B., Christoph, G., and Christian, D. (2009):** Diagnosing Schistosomiasis by Detection of Cell-Free Parasite DNA in Human Plasma. PLoS Negl Trop Dis. 2009 April; 3(4): e422.
2. **Hamilton, J.V., M. Klinkert and M.J. Doenhoff (1998):** Diagnosis of schistosomiasis: Antibody detection, with notes on parasitological and antigen detection methods. Parasitology 117: (suppl): S41-S45.
3. **Nash, T.E. and Deelder, A.M. (1985):** Comparison of schistosome excretory-antigens: Phenol sulfuric test active peak,

- cathodic circulating antigen, Gut associated proteoglycan and circulating anodic antigen. Am.J. Trop. Med. Hyg., 34:236.
4. **Luiz Carlos Pedrosa Valli, Herminia Yohko Kanamura, Paulo Cesar Cotrim, Guilherme Correa Oliveira and Edward Jose de Oliveira (2007):** Characterization of a clone from adult worm of cDNA library selected with anti-schistosome mansoni human antibodies dissociated from immune complexes: a preliminary report. Rev. Inst. Med Trop. S. Paulo vol. 49 no. 3 Sao Paulo May/June.
 5. **Abdeen, H.H., Attallah, A.F., Mansour, M.M., and Harrison, R.A. (1999):** Molecular cloning and characterization of the polypeptide backbone of circulating cathodic antigen. Mol. Biol. Biochem. Parasitol., 25:149.
 6. **Attallah, A.M., Yones, E., Ismail, H., El Masry, S.A., Tabll, A., Elenein, A.A. and El Ghawalby, N.A. (1999):** Immunochemical characterization and diagnostic potential of a 63-kilo Dalton *Schistosoma* antigen. Am. J. Trop. Med. Hyg., 60:493.
 7. **Sulbaran, G.S. Ballen, D.E., Bermdez, H., Lorenzo, M., Noya, O. and Cesari, M. (2010):** Detection of the Sm31 antigen in sera of *Schistosoma mansoni* – infected patients from a low endemic area. Parasite Immunology, 2010, 32, 20–28
 8. **Basch, P.F. (1981):** Cultivation of *Schistosoma mansoni* in vitro. I Establishment of cultures from cercariae and development until pairing. J. Parasitol., 67: 179-185.
 9. **Lee, W.K., Shalaby, A.K., Thakur, A., Medhat, M.A., Karim, M.A. and LoVerde, P.T. (1995):** Cloning of the gene for phosphoglycerate kinase from *Schistosoma mansoni* and characterization of its gene product. Molecular and Biochemical Parasitology 71: 221-231.
 10. **Sambrook, J., Fritsch, E.F. and Maniatis (1989):** Molecular cloning A laboratory manual 2nd. ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
 11. **Towbin, H., Staehlin, T. and Gordon, J. (1979):** Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and application. Proc. Natl. Acad. Sci., USA 76: 4350.
 12. **Beall, A. J. and Mitchell, F. G. (1986):** Identification of a particular antigen purified from selected portions of western blot. Journal of Immunological Methods 86: 217 – 223.
 13. **Ciselle, M. Knudsen, Katalin, F. Medzihradzsky, Kee-Chong lim, Elizabeth Hansell and James, H.M. (2005):** Proteomic analysis of *Schistosoma mansoni* cercarial secretions. Molecular and Cellular Proteomics 4: 1862 – 75.
 14. **Chitsulo, L., LoVerde, P. and Engles, D. (2004):** Schistosomiasis. Nat. Rev. Microbiol., 2: 12 – 13.
 15. **WHO (2002):** Strategic Direction: Schistosomiasis,

- World Health Organization, Geneva.
16. **Paulo, M., Arnon, D., Aureo, A., and Naftale, K. (2009):** Use of a saline gradient for the diagnosis of schistosomiasis. Mem. Inst. Oswaldo Cruz vol.104 no.5 Rio de Janeiro.
 17. **WHO-World Health Organization (2006):** Report of the scientific working group meeting on schistosomiasis for the special programme for research and training in tropical diseases. In A Rabello, M Enk (eds.), Progress towards the detection of schistosomiasis, WHO, Geneva, p. 67-71.
 18. **Polman, K., F. F. Stelma, S. Le Cessie, S. J. Devlas, S. T. Falcao Ferreira, A.M. Deelder and B. Gryseels (2002):** Evaluation of the patterns of *S. mansoni* infection and reinfection in Senegal, from faecal egg counts and serum concentrations of circulating anodic antigen. Ann. Trop. Med. Parasitol., 96: 679-689.
 19. **Doenhoff, M.J., P.L. Chiodini and J.V. Hamilton (2004):** Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? Trends Parasitol., 20: 35-39.
 20. **Van Lieshout, L., Polderman, A.M. and Deelder, A.M. (2000):** Rapid diagnosis of schistosomiasis by determination of circulating antigens CAA and CCA, in particular in individuals with recent or light infections. Acta Trop., 77: 69-80.
 21. **Legesse, M. and Erko, B. (2008):** Field-based evaluation of a reagent strip test for diagnosis of schistosomiasis mansoni by detecting circulating cathodic antigen (CAA) in urine in low endemic area in Ethiopia. Parasite 15(2):151-5.
 22. **Hassan, M.M., M.H. Hegab, S.Z. Soliman, O.A. Gaber, M.M. Shalaby and F.M. Kamel (1999):** Relationship between circulating antigen level and morbidity in *S. mansoni* – infected children evaluated by ultrasonography. Am. J. Trop. Med. Hyg., 61: 635-638.
 23. **Van Lieshout, L., De Jonge, N., El Masry, N. D., Mansour, M.M., Krijger, F. W. and Deelder, A.M. (1992):** Improved diagnostic performance of the circulating antigen assay in human schistosomiasis by parallel testing of circulating anodic and cathodic antigens in serum and urine. Am. J. Trop. Med. Hyg., 47: 463-469.
 24. **Pardo, J., Carranza, C., Turrientes, C.M., Perez, L.J., Lopez Velez, R., Ramajo, V. and Muro, A. (2004):** Utility of *Schistosoma bovis* adult worm antigens for diagnosis of human schistosomiasis by Immunosorbent Assay and electroimmunotransfer blot techniques. Clin. Diagn. Lab. Immunol., 11 (6): 1165-70.
 25. **Van Gool, T., Vetter, T., Vervoort, M.J., Doenhoff, J., Wetsteyn and D. Overbosch (2002):** Serodiagnosis of imported schistosomiasis by a combination of commercial

- indirect hemagglutination test with *S. mansoni* adult worm antigens and an enzyme linked immunosorbent assay with *S. mansoni* egg antigens. J. Clin. Microbiol., 40: 3432-3437.
26. **Annie, S., Yves, Jean Francois, Garin Arezki Izri, Caroline Verret, Pascal Delauny, Tom Van Gool and Francis Derouin (2005):** Development and evaluation of a western blot kit for diagnosis of schistosomiasis. Clin. Diagn. Lab. Immunol., 12 (4): 548-51.
27. **Valli, L.C.P., H.Y. Kanamura, R.M. DaSilva, R. Riberro-Rodrigues and A.D.R. Dietze (1999):** *Schistosoma mansoni*: Immunoblot analysis to diagnose and differentiate recent and chronic infection. Am. J. Trop. Med. Hyg., 61: 302-7.
28. **Al-Sherbiny, M.M., A.M. Osman, K. Hancock, A.M. Deelder and V.C. Tsang (1999):** Application of immunodiagnostic assays, detection of antibodies and circulating antigens in human schistosomiasis and correlation with clinical findings. Am. J. Trop. Med. Hyg., 60: 960-966.
29. **Balloul, J.M., Pierce, R.J., Gryzch, J.M. and Capron, A. (1985):** In vitro synthesis of a 28 kDa antigen present on the surface of the schistosomulum of *Schistosoma mansoni*. Mol. Biochem. Parasitol., 17: 105.
30. **Tarrab-Hazdai, D.A., Levi-Schaffer, F., Brenner, V., Horowitz, S., Eshhar, Z. and Arnon, R. (1985):** Protective monoclonal antibody against *Schistosoma mansoni*: antigen isolation, characterization and suitability for active immunization. J. Immunol., 135: 2772.
31. **Attallah, A.M., El Masry, S.A., Ismail, H., Abdel Aziz, M., Shehatta, A.S., Tabll, A., Soltan, A. and El-Wassif, A. (1998):** Immunochemical purification and characterization of a 74.0 kDa *Schistosoma mansoni* antigen. J. Parasitol., 84: 301.

تعريف الانتجين ذو الوزن الجزيئى ٣٨ كيلودالتون فى بول المرضى المصابين بمرض البلهارسيا المعوية المزمن باستخدام الجسم المضاد أحادى النسيلة والمزاح من غشاء النيتروسيليلوز. طريقة لتشخيص المرض

سمير محجوب

قسم الكيمياء الحيوية – كلية الطب – جامعة المنيا

مرض البلهارسيا المعوية من الأمراض المزمنة التى تصيب ٢٠٠-٣٠٠ مليون شخص فى جميع أنحاء العالم. لذا فإن الأبحاث تركز على تعريف وتوصيف الأنتيجينات التى يمكن أن تستخدم كمصل للمرض وكذلك لها قيمة تشخيصية. فى هذه الدراسة تم التعرف على أحد بروتينات السطح الخارجى للطفيل المسبب للمرض له وزن جزيئى ٣٨ كيلودالتون باستخدام مصل الدم من مرضى البلهارسيا المعوية المزمن. وباستخدام تقنية لطخة ويسترن تم نقل بروتينات مصل الدم وبول المرضى المصابين بالبلهارسيا المعوية إلى غشاء النيتروسيليلوز الذى تم حضائته مع الجسم المضاد أحادى النسيلة والمزاح من غشاء النيترو سيليلوز بطريقة كيميائية. الجسم المضاد أحادى النسيلة تعرف فى تجربة لطخة ويسترن على الأنتجين ذو الوزن الجزيئى ٣٨ كيلو دالتون فى بروتينات مصل الدم وكذلك بروتينات البول لمرضى البلهارسيا المعوية المزمن. ولكن لم يتعرف على أية بروتينات فى عينات البول المأخوذة من الأفراد الغير مصابين بالمرض وكذلك بروتينات بكتيريا E-coli. وحيث ان هذا الأنتجين يفرز فى بول مرضى البلهارسيا المعوية وامكن تعيينه باستخدام الجسم المضاد احادى النسيلة لذا يمكن استخدامه فى تشخيص المرض.