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COMPARATIVE STUDY ON IMMUNOBLOT VERSUS PCR IN DIAGNOSIS OF SCHISTOSOMIASIS *MANSONI* IN EXPERIMENTAL INFECTED MICE

By

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Abstract

This study compared PCR and Western blot techniques in diagnosis of schistosomiasis *mansoni.* Forty Swiss albino mice were used, thirty two mice were infected with cercariae of *S. mansoni* and eight mice were kept uninfected which were used as a control. Blood was obtained from four infected mice weekly beginning from the 1st week to the 8th week post infection. The study found that PCR was positive from the first week post infection, while Western blot technique was positive from the second week post infection. Thus, PCR diagnosed schistosomiasis *mansoni* earlier than Western blot technique, but both were able to diagnose.

Key words: Egypt, *Schistosoma mansoni*, PCR, Western blot technique.

Introduction

Schistosomiasis is a major parasitic disease that affects more than 200 million people worldwide, with more than 600 million people in the tropics are at risk for developing Schistosomiasis (CDC, 2015). *S. mansoni* top the list of Egyptian endemic parasitosis (Kilany *et al*, 2009). The early diagnosis is essential to start treatment, and avoiding the complications of chronic schistosomiasis (Massoud *et al*, 2010; Ismail *et al*, 2016). Sabry *et al.* (2015) reported that their study was one of few, addressing the possibility of direct relation between *S. mansoni* & hepatic carcinoma, concluding an initial indication of equal risk value of both human chronic *S. mansoni* infection and HCV infections in precipitating hepatocellular carcinoma among Egyptian patients. Schistosomiasis diagnosis requires viable eggs in fecal samples (direct methods). Despite 100% specificity of these direct parasitological methods, yet their sensitivity is rather poor even with concentration techniques. This may be explained by many factors such as day to day variation, uneven distribution of eggs in the excreta, small amount of excreta examined and light infection. Tissue retaining eggs due to fibrosis is another factor (Venderame *et al,* 2001).

Comparative studies of parasitological and serological methods confirmed higher sensitivity of latter one especially in little endemic area. The existence of cross reactivity and low specificity post-treatment due to reduction of specific antibody titer were great disadvantage of immunodiagnostic techniques (Pontes *et al,* 2003). PCR and western blot technique are extremely sensitive and specific technique with widespread use in diagnosis of infectious diseases, particularly with *S. mansoni* (Kane, 2006).

This study valuated polymerase chain reaction (PCR) and western blot technique (WB) in early on in diagnosis of *Schistosoma mansoni* in experimental infected mice

Materials and Methods

An experimental study was performed on forty Swiss albino mice divided into two groups: 32 mice were infected with *S. mansoni* 60 cercariae via tail and 8 mice were noninfected control group. One week post infection 4 mice of the infected group and one mouse of control group were sacrificed by cervical dislocation. Blood from each mouse was collected and divided in to two

parts (one part preserved in EDTA for PCR and second centrifuged to obtained serum for Western blot technique) which stored at -20°C. This step was repeated every week for 8 weeks post infection. Cercariae and adult worms of *S. mansoni* (crude antigen for Western blot technique) were obtained from Schistosome Biological Supply Program Unit (SBSP), Theodor Bilharz Research Institute. Genomic DNA was extracted from frozen blood samples by DNA extractor Kit (PAXgene®, PreAnalytiX® (PreAnalytiX GmbH); QIA GEN® (QIAGEN Group); BD VACUTAI- NER®, BD Hemogard™, Safety Lok™ (Becton Dickinson and Company, Franklin Lakes, NJ, USA); Styrofoam® (Dow Chemical Co.) according to manufacturer's instructions. Extracted DNA was amplified by PCR targeting Schf111-gene, using set of primers: Schf111 (5' CGA TC AGGACCAGTGT TCAGC-3') and Schr111 (5' GACAGGTCA ACAAGACGAACTCG-3') after (Gomes, 2006), amplify a 110-bp fragment Reaction mixture and conditions were done in a total volume of 25µl. Amplified products were visualised with 1.5% agarose gel electrophoresis after ethidium bromide staining. Western blot technique determined anti *S. mansoni* antibodies in infected mice sera against specific protien bands of *S. mansoni* adult crude antigens by using enzyme linked immunotransfer blot (EITB). Adult antigen was prepared (Deelder *et al,* 1976). Fresh extracted *S. mansoni* adult were washed several time and homogenized with (0.01M) phosphate buffered saline (PBS) (pH 7.4), in a homogenizer at 6000r.p.m for 20 minute in ice bath and sonicated. Sonicated sample was subjected to high speed centrifugation 20.000 r.p.m. for one hour at 4°C.The protein content was measured (Lowry *et al*, 1951) and then stored at -20°C until used. At first, the molecular weight of *S. mansoni* antigens was determined by using SDS-PAGE in which gel was soaked overnight in coomassiae stain, destained with the destaining solution with several changes till bands became clear. Protein bands were scannered to determine molecular weights of *S. mansoni* antigen. Then electrophoretic transfer of proteins from Sodium Dodecyl Sulfate–Polyacryl amide Gel Electrophoresis (SDS–PAGE) to a nitrocellulose sheet was done. Nitrocellulose strips of fractionated *S. mansoni* adult crude antigen were used against the specific anti-*S. mansoni* mice sera at dilution 1:100 by EITB technique to determine immune response to protein bands. Reaction was read by Gel pro-analyzer 3.1.

Results

Table 1: Number of mice in which specific *S. mansoni* DNA of Schf111 gene detected by PCR.

Period post infection	PCR positive mice/examined mice
1st week	2/4
2nd week	2/4
3rd week	4/4
4th week	4/4
5th week	4/4
6th week	4/4
7th week	4/4
8th week	4/4

PCR targeting Schf111 gene detected DNA of in 50% of mice in 1st & 2nd weeks post infection and in 100% from third week to eighth week

Table 2: Bands of antibodies in infected mice sera against specific protein fractions weekly

Period post infection	Protien bands
1st week	No bands.
2nd week	Protein band at 32 KDa.
3rd week	Protein band at 32 KDa.
4th week	Protein bands at (32 and 43) KDa.
5th week	Protein bands at (32 and 43) KDa.
6th week	Protein bands at (32, 43, 95 and 130) KDa.
7th week	Protein bands at (32, 43, 95 and 130) KDa.
8th week	Protein bands at (32, 43, 95 and 130) KDa.

Western blot tests diagnosed schistosomiasis on second week post infection. Also, specific protein bands of adult crude antigen reacted with antibodies in sera increased by time.

Lanes 1 to 32 showed PCR results from 1st to 8th week post infection. Each lane represented one PCR on an infected mouse blood (Each four mice represented one week). Lane M: Molecular weight marker, Lane +ve: Positive control (110 bp), Lane -ve: Negative control,In 1st week, lane 1 and 3 positive but lane 2 & 4 negative, In 2nd week, lane 5 & 6 negative but lane 7 & 8 positive, In 3rd week, lane 9, 10, 11 & 12 positive, In 4th week, lane 13, 14, 15 & 16 positive, In 5th week, lane 17, 18, 19 & 20 positive, In 6th week, lane 21, 22, 23 & 24 positive, In 7th week, lane 25, 26, 27 & 28 positive & In 8th week, lane 29, 30, 31 &32 positive.

Fig.1: *Schistosoma mansoni* DNA in blood of infected mice by PCR

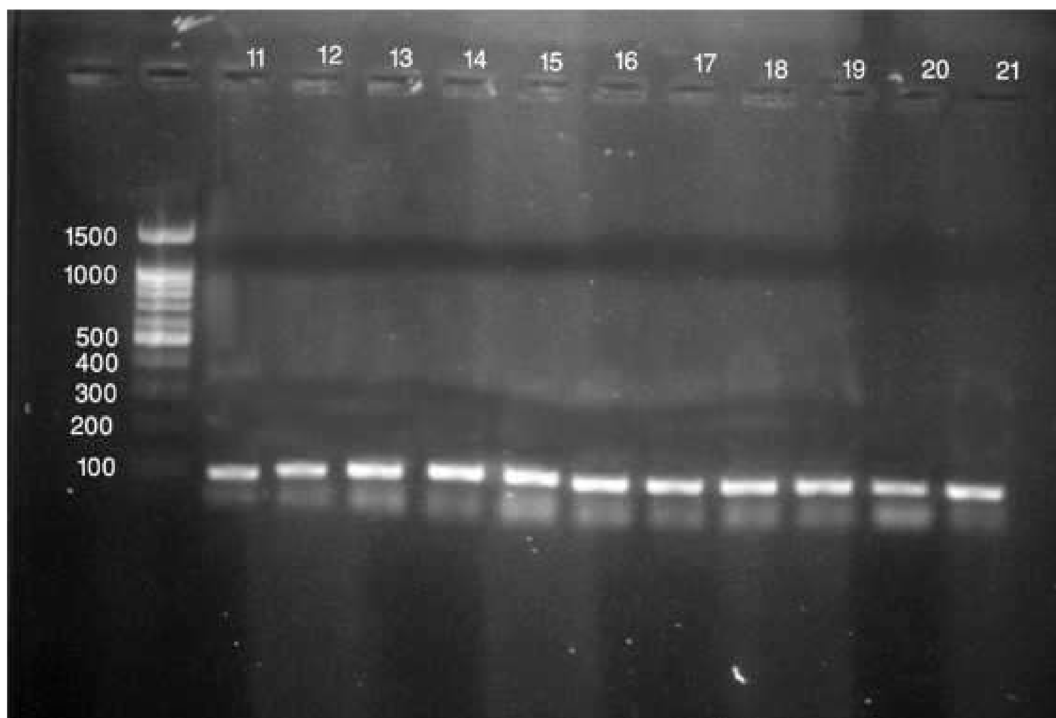

Fig. 2: *Schistosoma mansoni* DNA in blood of infected mice by PCR

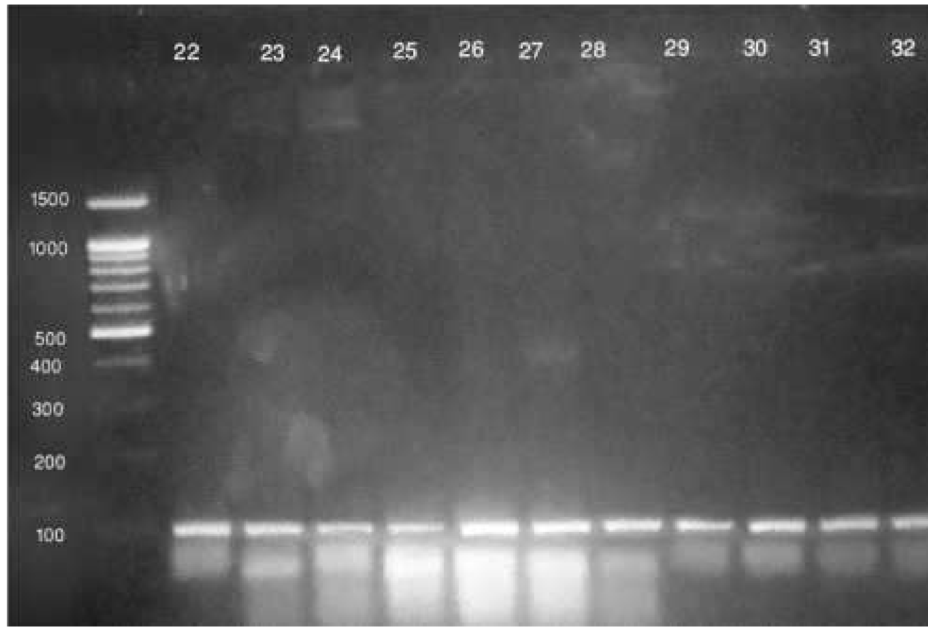

Fig. 3: *Schistosoma mansoni* DNA in blood of infected mice by PCR

SDS-PAGE of *S. mansoni* crude antigen stained with coomassiae stain shown (Fig. 4). Antigen was separated into 10 bands of molecular weight 140, 130, 118, 95, 74, 55, 46, 43, 32 and 11 KDa.

Protein bands of antigen were electro-transferred from SDS-PAGE to nitrocellulose sheet and reacted with serum of infected mice. Reaction appeared in 5 bands 130, 95, 55, 43 and 32 KDa.

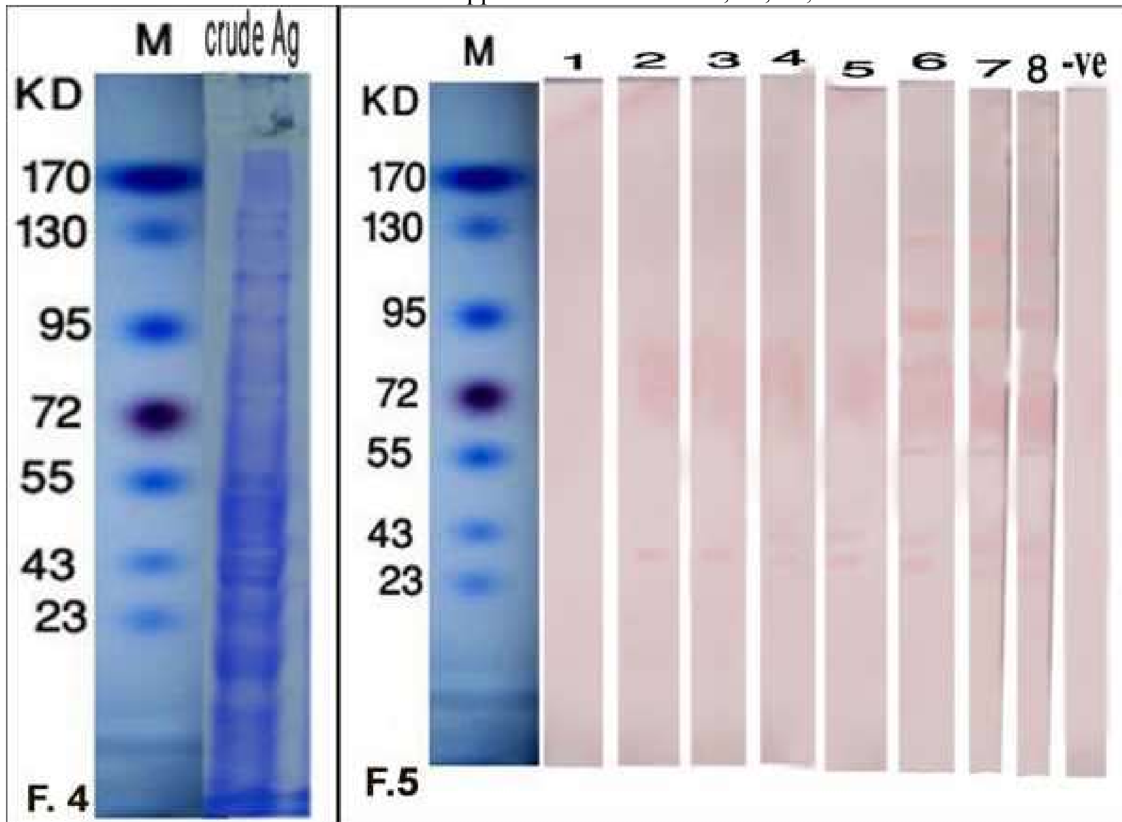

Fig. 4: SDS-PAGE characterization of crude antigen of *S. mansoni*

Fig. 5: Western blot technique characterization of crude antigen of *S. mansoni*

On the left side of image (5), there was (marker) and on right side of image (negative control). Columns 1 to 8 showed Western blot tests results changed from 1st to 8th week post infection. Each column represented one Western blot test (four mice each week)

Discussion

The present results agreed with Hamburger et *al.* (1991), Pontes *et al.* (2002) and Pontes *et al.* (2003). They reported high sensitivity of PCR in detecting patients with active intestinal schistosomiasis and that it was 10 times more sensitive than Kato-Katz examination. Furthermore, it was specific without cross-reaction with DNA from other helminthes. Also, Suzuki *et al*. (2006) detected *S. mansoni* DNA in mouse serum samples based on touchdown PCR and that *S. mansoni* DNA was detected in the sera at 2 weeks post-infection. These data suggested that touchdown PCR was a potential tool for the early diagnosis of *S. mansoni* infection. Sandoval *et al.* (2006) performed PCR on a *S. mansoni* murine experimental model, which permits follow up of the parasite from the acute to the chronic stage of infection. The results suggested that this new PCR-based approach could be useful for the detection of acute schistosomiasis. Also, Helmy (2007) detected *S. mansoni* DNA in mouse serum samples based on touchdown PCR (1-8 weeks post-infection). He reported that *S. mansoni* DNA was detected in sera at 2 weeks post infection. The results showed that PCR is useful in early diagnosis. Abdel-Hafeez *et al*. (2015) coincided with the present study as it indicated that PCR using patient's serum can detect circulating DNA of *S. mansoni* in 96.4% of active intestinal schistosomiasis. They concluded that PCR appears to be an effective diagnostic technique for *S. mansoni* infection, especially where a low worm burden exists, such as in chronic cases. The high degree of sensitivity of PCR may make it a good diagnostic alternative to Kato Katz method as PCR can diagnose recent infection in the early prepatent periods where there are no eggs to be detected by Kato Katz technique. Sadek *et al.* (2008) concluded that PCR in human serum can detect circulating DNA of *S. mansoni* in active intestinal schistosomiasis confirming its value in diagnosing infection in areas with low intensity of infection, and rendering it a more useful diagnostic alternative than Kato-Katz technique in the early prepatent period where there are no eggs to be detected. Also the higher degree of specificity of PCR may make it a better diagnostic alternative to serological techniques. So, the high sensitivity and specificity of PCR in detecting the *S. mansoni* circulating DNA makes it a promising method for the diagnosis of active early infection.

Oliveira *et al.* (2010) reported that PCR could be an important tool for detecting *S. mansoni* infection in individuals excreting few eggs in feces. Moreover, the determination of the infection through the detection of *S. mansoni* DNA in stool samples from seropositive individuals represents a new means of confirming the results of IgG-ELISA for schistosomiasis.

Regarding Western blot technique, the present results agreed with Simpsons *et al.* (1983) who recognized an antigen of molecular weight of 32KDa on the surface of *S. mansoni* schistosomula by antibodies obtained from infected mice. Besides, Chappell and Dresden (1988) reported that among worm proteins separated by electrophoresis in SDS, only a limited molecular weight region (32-35 KDa) appeared to constitute the major immunogens in *S. mansoni* infected mice. Evengard *et al.* (1990) reported anti *S. mansoni* adult worm IgG antibodies against protein bands at 32- 35 KDa, when analyzing sera from patients in an early stage of *S. mansoni* with 100% sensitivity. Valli *et al.* (1999) performed a study for diagnosis and differentiation between recent and chronic infection with schistosomiasis *mansoni* by immunobloting technique. They found that *S. mansoni* soluble adult worm antigen preparation had an immunogenic fraction with a molecular weight of 31 & 32 KDa (Sm31/

32). Both cathepsin B (31 kD) and asparaginyl endoproteinase (32 kD) were capable of inducing an early and intense immune response detected approximately 4 weeks after infection.

The present results coincided with Soliman *et al.* (2003) who used SDS- PAGE to separate soluble worm antigen preparation of *S. mansoni*. The polypeptide of that antigen was recognized at 32KDa. Also, Noya *et al.* (2003) recognized protein band at 32KDa from adult worm antigen by Western blot technique. Evengard *et al.* (2008) reported that on immunoblots, using a freeze-dried adult worm antigen of *S. mansoni,* IgG1 and IgG3 antibodies were recognized by antigens at 32-35 kDa. Antibodies against these antigens could be considered as a marker of early infection in previously non exposed visitors to endemic areas.

Sulahian *et al.* (2005) used western blot technique for analysis of the crude extract of *S. mansoni.* Six immuno-dominant bands (65, 70, 80, 95, 110 and 120 KDa) were revealed with sera from patients with schistosomiasis. The authors considered the presence of three or more bands in the range 65 to 120 KDa id diagnostic for *Schistosoma* infection and had specificity of 100%. However, other studies showed disagreed with Ruppel *et al.* (1985) who reported that antibodies against proteins were detected at 67 KDa were pronounced in all mice infected with *S. mansoni*. Noya *et al* (1995) who used immunoblot technique to evaluate *S. mansoni* adult worm antigen found that eight highly specific polypeptide molecules from the parasite at 118, 114, 105, 74, 71, 45, 36 and 30 KDa were recognized by total IgG. Shaheen *et al.* (1996) identified low molecular weight antigenic fractions (30-40 KDa), in soluble worm antigen preparation, as the most strongly recognized bands by IgG1 and IgG3 in human sera with prepatent infection with *S. mansoni.* Attallah *et al.* (1998) detected a polypeptide antigen at 74 KDa in the antigenic extracts of *S. mansoni* adult worms by Western blot technique. Also, Abath *et al.* (2000) identified 13 KDa protein band in the tegument of adult worms by Western blot technique Sulahian *et al.* (2005) reported that For serologically proven cases, the sensitivity of WB analysis was 97.3%. The overall sensitivity and specificity for both groups of patients were 89.5 and 100%, respectively, with positive and negative predictive values of 100 and 91.3%, respectively. They concluded that WB analysis is a useful technique for the immunological diagnosis of schistosomiasis. Abdel-Ghany *et al*. (2015) in Egypt schistosomiasis is a chronic disease with considerable social impact. They added that despite the availability of affordable chemotherapy, drug treatment did not significantly reduced the overall number of disease cases.

Conclusion

The outcome data showed that PCR diagnosed schistosomiasis *mansoni* in the first week post infection, while Western blot technique from the second week post infection, so both techniques diagnosed the infection during the prepatent period. PCR is a far more sensitive diagnostic technique (especially for cases with low worm burdens such as chronic infection cases) for the detection of *S. mansoni* infection

Conflict of interest: The authors declare that they have no conflicts of interest. All authors contributed in the activities in this study on their own behalf and did not receive any grant.

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