PCR IN DIAGNOSIS OF SCHISTOSOMA MANSONI-IN MICE

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ABSTRACT

Schistosomiasis is the second most important human parasitic disease in the world after malaria. It's a chronic and debilitating parasitic disease that affects approximately 200 million people in the developing world. The aim of this study was to evaluate the efficiency of PCR technique in the early diagnosis of Schistosomiasis and study the effect of Schistosoma mansoni infection on selected biochemical parameters. This study was conducted on 40 mice. Eight clinically-healthy mice were served as a control group (group I) and 32 S.mansoni-infected mice were divided according to the period of infection by S.mansoni into four equal groups (eight mice for each); groups II, III, IV and V were infected for 2 weeks, 1 month, 1.5 months and 2 months respectively. Our results revealed that there was a significant increase in the serum ALT and AST activities at 2 weeks, 1 month and 1.5 months post-infection followed by a significant decrease at 2 months in comparison with the control group. Moreover, there was a significant increase in the serum total protein levels and a significant decrease in the serum albumin levels in all infected groups. Moreover there was a significant increase in MDA and G-SH levels with increasing period of infection, however, catalase activity showed the opposite results. Regarding, PCR results: real time PCR has been detected the S. mansoni infection at 2 weeks, so it is the best method for early detection of S.mansoni in serum according to this study.

Keywords: *S. mansoni, Mice, Antioxidant enzymes, PCR*

INTRODUCTION

Schistosomes are blood flukes that inhabit the portal blood system of many mammalian species. Schistosomiasis is a major cause of debilitating illness in the world with approximately 10% of infected persons suffering chronic disease with significantly impaired liver functions. The number of people infected with liver fluke in the world has been estimated to be between 200 and 300 million resulting in the mortality of approximately 200,000 people per year **(WHO , 1995)**.

Biological antioxidants are compounds that protect biological systems against the potentially harmful effect of oxidant products. Antioxidants are effective as they give their own electron to free radicals. When a free radical gains this electron, it will no longer need to attack the cell as the chain reaction of oxidant material has been broken. After donating an electron, the antioxidant becomes a harmless free radical because it has the ability to ac-

commodate the change in electrons without becoming reactive (**Dekkers et al., 1996**). **Thomas (2000)** classified antioxidants into two major categories; enzymatic antioxidants as superoxide dismutase, glutathione peroxidase, glutathione peroxidase, glutathione reductase and catalase, and non-enzymatic antioxidants as reduced glutathione and vitamin C.

It was reported that in parasitic diseases, there was a complex and dynamic physiological relationship between parasite and host antioxidant defense components (**Connors et al., 1995**). In schistosomiasis, granuloma macrophages isolated from hepatic, intestinal, and pulmonary lesions were found to release significant amounts of O.2- and H_2O_2 radicals (**Gharib et al., 1999**). PCR is an extremely sensitive and specific technique with wide spread use in the diagnosis of infectious diseases, it was evaluated as a diagnostic tool for detecting infection of humans with S. mansoni (**Pontes et al., 2002**).

MATERIAL & METHODS

1. Animals and Infective stage:

Fourty male Swiss albino mice aged between 6 and 8 weeks were bred and maintained at the experimental animal research unit of the Schistosoma biological supply program at Theodor Bilharz Research Institute (TBRI, Giza, Egypt). These mice were divided into five equal groups (eight mice for each); group I was clinically healthy and served as a control, group II was infected for two weeks, group III was infected for one month, group IV was infected for 1.5 month and group V was infected for 2 months. Mice were kept on a standard commercial diet and provided with water ad libitum.

The infective stage of Schistosoma (S. mansoni cercariae) was obtained from parasitological department of (TBRI, Giza, Egypt). Mice were infected with an Egyptian strain of S. mansoni (80 ± 10 cercariae/mouse) using the body immersion technique.

2. Sampling and biochemical analysis:

Blood samples were collected from each animal by decapitation. Each blood sample was collected in a test tube without anticoagulant for separation of serum. Samples were centrifuged at 3000 rpm for 10 minutes and kept at -20°c until biochemical analysis of Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) activities according to (**Murray et al., 1984**) , total protein (T.P) according to (**Kollar, 1984**) and albumin levels according to (**Kaplan et al., 1984**) by using commercial kits (Diamond diagnostics, Egypt) as instructed by the manufacturer. Liver tissue samples were also collected from each mouse and stored frozen at -20°C and used for analysis of tissue MDA according to (**Yoshioka et al**), tissue catalase according to (**Aebi, 1984**) and reduced Glutathione (G-SH) according to (**Beutler, 1963**) by using commercial kits (Biodiagnostic Company,cairo) as instructed by the manufacturer respectively.

3- PCR:

3.A. Extraction of DNA:

DNA were extracted through a Gene JET Genomic DNA Purification Kit (Fermentas Company).

3.B.Touch down PCR technique:

Touch down PCR technique was performed

according to **(Don et al., 2006)**.

4. Statistical analysis:

These result were analyzed by SAS computer program according to **Snedcor and Cochran (1989)**.

RESULTS

Mice infected with S. mansoni under this investigation showed significant increase in ALT and AST activities a after 2 weeks, 1 month and 1.5 months post-infection followed by a significant decrease after 2 months of infection. Moreover, serum total protein levels were significantly increased in all infected groups in comparison with the control one. However, there was a significant decrease in albumin level in all infected groups in comparison with the control group (Table 1). Regarding, MDA and G-SH activities there was a significant increase in their levels with increase the period of infection in comparison with control group. In contrast, catalase activity showed a significant decrease (Table 2).

DISCUSSION

It was previously reported that in parasitic diseases there is a complex and a dynamic physiological relationship between the parasite and the antioxidant defense components of the host **(Connors et al., 1995)**. **Pascal et al. (2000)** reported that the infection with S. mansoni not only triggers the production of reactive oxygen species, but also leads to the alteration of the antioxidant defense mechanism.

The obtained tissue MDA results were in accordance with that previously reported by **Shaheen et al., (1996); Dessein et al., (1999); Pascal et al., (2000)** showed that lipid peroxides were elevated by S. mansoni throughout the different durations of infection. This is coincided with the phenomenon of that production of free radicals in the chain of biochemical reactions results in an increase in lipid peroxides. It should be pointed out that oxidative stress due to schistosomiasis causes the same effect, since fibrosis associated with the disease is stimulated by reactive end products of lipid peroxidation. Reduced glutathione has a role in the free radical scavenging and also act as a cofactor in the chemical structure of several enzymes and has a role as non -toxic storage form of cystiene working as a defensive agent against oxidizing molecules, and potentially harmful xenobiotics such as heavy metals (Elia et al., 2003). In the present study there was a significant increase in tissue GSH after 1, 1.5 and 2 months of S. mansoni infection. Results of glutathione content in infected mice livers revealed a highly significant reduction resulting from an oxidative stress due to schistosomiasis. Such depletion might be caused by increased cytotoxicity with H_2O_2 which leads to inhibition in glutathione reductase, the latter responsible for keeping glutathione in its reduced state. These results are in accordance with earlier reports **(Lew et al., 1985; Hirota et al., 1989; Song et al., 2000)** who showed that hepatic tissue represents the major GSH reservoir for extra-hepatic levels and both hepatic and extra-hepatic GSH release into the circulation is facilitated by stressors through an alpha receptor mechanism. The stress-induced efflux of GSH is important because blood GSH can be used directly for detoxification of peroxides through the action of blood-borne GPX. In contrast, other studies have

reported opposite findings as mentioned by **Yegen et al.. (1990); Amaiz et al. (1995).**

Tissue catalase levels, the results in this investigation came in accordance with the findings of **Dessein et al., (1999)** who documented that tissue catalase levels were decreased during first (4 and 5) weeks post-infection. It means that catalase is affected before deposition of parasite eggs in the liver, this depletion is critical as shown by the increased cytotoxicity of H_2O_2 which formed from dismutation of O-2 by SOD enzyme and accumulate in endothelial cells **(Hara et al., 2002).** H_2O_2 must be metabolized to innocuous products by catalase to prevent it from being converted to OH- radical, so the level of catalase enzyme was markedly decreased in mice infected with S. mansoni **(Song et al., 2000).** ALT and AST activities are in agreement with previous studies **(Awadalla et al., 1975; EL Aasar et al., 1989; Gharib et al., 1999; ElSokkary et al., 2004).** The authors attributed the increase of transaminase enzyme activities in serum of S .mansoni-infected mice after 1, 1, 5 months to increased hepatocellular damage, decreased hepatocytes' population, and increased cell membrane permeability, which lead to release of transaminase enzymes into the circulation. They also attributed the decrease in transaminase enzyme activities after 2 months of infection to complete destruction of hepatic cells, which are replaced by fibrous connective tissue and subsequent decrease in transaminase production.

The elevation in serum total protein levels under this investigation were in agreement with **Clayton and Frank, (2004)** who attributed the increase in serum total protein to increased globulin fraction due to the reactivity of immune body defense in response to parasitic infection. The increased globulin fraction results in a reversal of A/G ratio in infected mice and subsequent increase in the serum total protein. In addition, **Amin and Mikhail (1989)** found that during S. mansoni infection, the serum protein fractions showed marked alteration. These changes developed during maturation of worms and become more evident with initiation of egg deposition. On the other hand, our result were disagree with an earlier study by **Mousa et al. (1975)** who found that in hepatic fibrosis as a result of schistosoma infection, there was a significant decrease in total protein . The author attributed the decrease in serum total protein to decreased protein anabolism, increased protein catabolism, as well as amino acid malabsorption. Serum albumin levels were in accordance with previous studies **(EL Aasar et al., 1989; Gharib et al., 1999; El Sokkary et al., 2002)** who attributed decreased serum albumin levels to complete destruction of hepatic cells, which are replaced by fibrous connective tissue. The hypoalbuminemia may also be due to inhibition of albumin anabolism and increased albumin catabolism **(Coles, 1986).**

Diagnosis of Schistosomiasis is usually based on the detection of parasite eggs by fecal examination or based on immunological methods. The fecal examination is simple, highly specific and cost-effective. Therefore, it is routinely used for epidemiological studies and for control programs in endemic areas **(Katz et al., 1972 and Bowie et al., 2004).** However, it often misses light infections **(Engels et al., 1996)** and is not able to detect the

infection until the parasite begins to lay eggs. One of the main requirements for diagnosing schistosomiasis is the development of a more sensitive method. Since PCR is an extremely sensitive and specific technique with wide spread use in the diagnosis of infectious diseases, it was evaluated as a diagnostic tool for detecting infection of humans with S. mansoni **(Pontes et al., 2003)**.

PCR assay is a sensitive and specific tool for the identification of cercariae in water **(Hamburger et al., 1998)** and **(Hamburger et al., 2001)** and of infected snails. (**Pontes et al., 2002)** reported the detection of S. mansoni DNA in human serum and feces. They believed that the PCR assay might be a valuable alternative for the diagnosis of the disease. This new technique is expected to overcome the disadvantages of stool examinations and serological tests.

In this experiment we used a R.T touch-down PCR, mouse sera were used as templates **(Don et al., 2006)**. the touchdown PCR swept away unexpected spurious bands. Additionally, this method enabled us to assay serum samples directly. In fact, 2 µl of serum is enough as a sample using this method. Also a probe for the 121-bp highly repeated sequence of S. mansoni which used in this study did not react with S. haematobium DNA this result in accordance with **Hamburger et al., (1991)**.

In conclusion, PCR has a great value and seemed to be the best method for early detection of S.mansoni infection in the serum of affected patients starting from two weeks due to the presence of free circulating DNA and the use of this PCR assay may be poseible to detect schistiomiasis at an early stage of human cases. Thus PCR assay may prove a powerful tool for monitoring outbreaks in endemic area and the early diagnosis of patients coming back from affected countries.

Table 1: Effect of *S. mansoni* infection on serum ALT, AST activities, T.P. and albumin levels in infected mice:

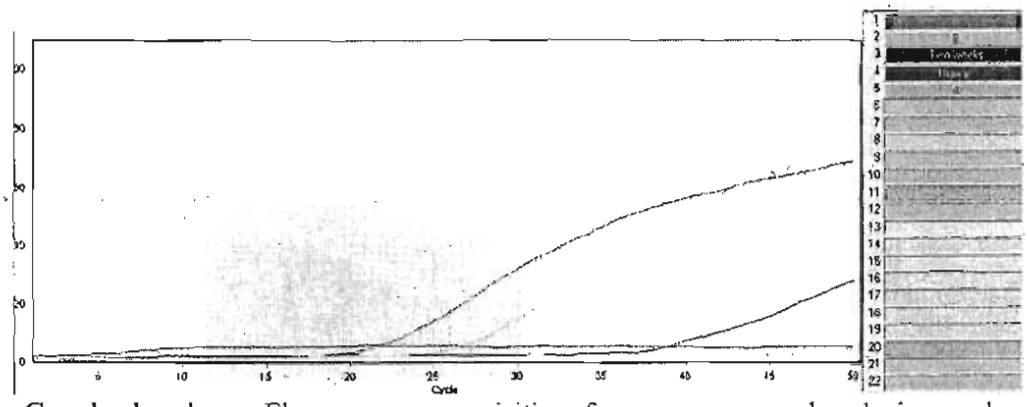
Parameters / Groups	ALT (U/L)	AST (U/L)	T.P. (g/l)	Albumin (g/l)
Group I (n = 8)	30.1 ± 2.8 [d]	31.6 ± 3.4 [d]	58 ± 1.4 [c]	48.5 ± 3.06 [a]
Group II (n = 8)	29.8 ± 0.5 [d]	33.2 ± 1.1 [d]	56.9 ± 1.1 [c]	46.7 ± 1.49 [a]
Group III (n = 8)	84.0 ± 4.1 [b]	140.2 ± 6.5 [b]	65.8 ± 3.2 [b]	46.4 ± 2.4 [a]
Group IV (n = 8)	94.5 ± 2.2 [a]	178.0 ± 7.8 [a]	69.2 ± 3.2 [b]	32.6 ± 2.5 [b]
Group V (n = 8)	78.8 ± 3.3 [c]	128.7 ± 8.7 [c]	87.2 ± 2.9 [a]	26.8 ± 3.1 [c]

[a,b,c,d] Different superscript letters in the same column means significantly different at $P < 0.05$.

Table 2: Effect of *S. mansoni* infection on tissue Catalase activity, G-SH and MDA levels in infected mice:

Parameter / Groups	Catalase (U/g)	GSH (mg/g)	MDA (mg/g)
Group I (n = 8)	1.05 ± 0.1 [a]	12.30 ± 1.4 [d]	0.2913±.10204 [d]
Group II (n = 8)	1.06 ± 0.08 [a]	12.60 ± 1.07 [d]	0.5263±.04596 [c]
Group III (n = 8)	0.48 ± 0.041 [b]	20.90 ± 2.10 [c]	0.6675±.06541 [b]
Group IV (n = 8)	0.38 ± 0.036 [c]	54.10 ± 6.04 [b]	1.4725±19797 [a]
Group V (n = 8)	0.26 ± 0.027 [d]	87.90 ± 6.90 [a]	1.8263±.07463 [a]

[a,b,c,d] Different superscript letters in the same column means significantly different at $P < 0.05$.



Graph showing: Flourescence acquisition from serum samples during real time PCR cycling the CT values of 2 months infected mice serum samples (red line) was the lowest followed by 1 month infected mice serum sample (yellow line) then 2 weeks infected mice serum sample (blue line) in addition human serum sample was included as negative control (violet line). The CT value is the number of cycles required for the fluorescent signal to cross the threshold. CT values are inversely proportional to the amount of target nucleic acid.

CT values ≤ 29 are strong positive .

CT values 30-39 are moderate positive .

CT values ≥ 40 are weak reaction.

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الملخص العربى

تفاعل البلمرة المتسلسل فى تشخيص بلهارسيا المستقيم فى الفئران

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قسم الكيمياء الحيوية وكيمياء التغذية

كلية الطب البيطرى - جامعة المنصورة

اجريت هذه الدراسة على عدد 40 فأر، 32 فأر مصاب بالبلهارسيا تم تقسيمهم الى 8 فئران مصابة لمدة اسبوعين ، 8 فئران مصابة لمدة شهر، 8 فئران لمدة 1.5 شهر و 8 فئران لمدة شهرين هذا بالاضافة الى 8 فئران غير مصابة كمجموعة ضابطة . وقد اجريت هذه الدراسة لمعرفة تأثير الاصابة بالبلهارسيا على بعض القياسات البيوكيميائية (نشاط انزيم الالانين امينو ترانسيفيرز، نشاط انزيم الاسبرتيت امينو ترانسيفيريز معدل الجلوتاثيون المختزل نشاط انزيم الكتاليز، مستوى البروتينات، مستوى الالبيومين) وقد اتضح من هذه الدراسة مايلى: زيادة نشاط انزيم الالانين امينو ترانسيفيريز و نشاط انزيم الاسبرتيت امينو ترانسيفيريز فى مصل الفئران المصابة لمدة اسبوعين، شهر ، شهر ونصف على التوالى مع خمول نشاطهم فى مصل الفئران المصابة لمدة شهرين مع زيادة مستوى البروتينات ونقص مستوى الالبيومين فى مصل الفئران المصابة لمدة اسبوعين، شهر، شهر ونصف و شهرين على التوالى بالمقارنة مع المجموعة الضابطة هذا بالاضافه الى زيادة نشاط انزيم الكتاليز و معدل الجلوتاثيون المختزل مع زيادةنشاط المالون داى الدهيد فى خلايا كبد الفئران المصابة .

مما سبق يتضح كفاءة تفاعل البلمره المتسلسل فى التشخيص المبكر لبلهارسيا المستقيم