

Detection and Quantification of Pro – Inflammatory Cytokine in Sera and Urine of Sudanese Patients Infected with *Schistosoma Haematobium*

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Abstract

Human infections with Schistosomiasis and other helminths induce strange immune responses which are characterised by the production of Th1 and Th2 cytokines. One hundred and thirty sera and urine were collected from patients with urinary schistosomiasis in two villages south Elduiem (Sudan). The disease were confirmed by finding *Schistosoma haematobium* eggs in urine using syringe filtration techniques. 70 individuals who were *Schistosoma haematobium* negative included in the study as controls 42 from endemic area and 28 from non-endemic area were tested IFN γ and IL 2 were found in urine and sera. There are a positive association between the production of cytokine and intensity of the infection. They are low intensity in control of endemic area which can be used for early detection of the disease in endemic area.

Keywords ELISA, *Shistosoma haematobium*, IL2, Pro-inflammatory cytokine.

Introduction

Schistosomiasis is one of the wide spread of all parasitic infection of man. The (WHO 2006) estimated that Schistosomiasis and soil transmitted helminths represent more than 40% of the global disease burden caused by all tropical diseases excluding malaria. Urinary Schistosomiasis caused by *Schistosoma haematobium* is endemic in 53 countries in the Middle East and most of the African continent Chitsulo, et al. especially in the rural areas where only the surface water bodies are sources of water supply [1,2]. *Schistosoma haematobium* infects over 112 million individuals and results in 150,000 deaths annually in sub-Saharan Africa [3]. In a study from Kenya, individuals with chronic infections and hepatosplenic disease appear to have predominantly Th1 responses to Schistosoma antigens and more severe pathology [4]. T Milner et al. in his study from Schistosoma-endemic area in Zimbabwe showed that egg positive people had significantly higher levels of specific antibodies, IL-2, IFN- γ and IL-23 [5]. In contrast, egg-negative individuals had significantly higher circulating IL-10, IL-4, IL-13, and IL-21 that were detected with high frequency in all participants.

Aim of the Study

Since World Health Organization (WHO) recommends that infection levels are determined prior to designing and implementing

control programmes, as the treatment regimens depend on the population infection prevalence and the sensitivity of the parasitological infection diagnostic method is less reliable when infection levels are low. In this study we aimed to compare between parasitological methods and serological ones in diagnosing infected individuals in an endemic area.

Ethical Consideration

Ethical and institutional approval for the study was obtained from the medical research council of the National Ribat University. Permission for initiation of the study in the area was obtained from the Health Services Director (Eldwam locality). Objectives and methods were clearly explained to the community. Oral consent was obtained from the participants and parents/guardian before sampling.

Materials and Methods

Study design

This is a cross-sectional and case control study conducted to study Pro-inflammatory cytokines in sera and urine of Sudanese patients infected with *Schistosoma haematobium*.

Study area

This study was conducted in Eldwam locality which is approximately 120 km south to Khartoum. It is a well-known *Schistosoma haematobium* endemic zone in White Nile state in Sudan.

The principle source of water is White Nile river and there are large farms which grow sugar cane for sugar industry, also grow maize, wheat and vegetables. Fishing is carried in the White Nile. Temporary pools are created by the over flow of the White Nile during the rainy season. This locality was chosen because there is no other helminthic infections and low *Schistosoma mansoni* prevalence [6-8].

Study population

A total of 130 Sudanese patients from Eldwam locality whom were tested positive for *Schistosoma haematobium* eggs in urine were recruited in this study. 42 persons from the same locality whom were tested negative for *Schistosoma haematobium* eggs in urine and 28 healthy controls from a non-endemic area. Samples were collected during January 2014.

Sampling and Procedures

Urine and Stool collection and examination

A single terminal urine samples (20-50 ml) was collected in 50 ml containers from each individual of the study population. The samples were obtained between 10:00 am and 14:00 pm. Few drops of saponin solution were added to the samples with visible hematuria to enhance clarity in microscopy [9]. The specimens were appropriately labelled with identification numbers and placed in cold box with ice packs. They were processed 1-2 hrs after collection in the field. 10 ml was filtered through a 25 mm nucleopore filter (12 µm pore size) [10]. The filter was examined microscopically for *Schistosoma haematobium* eggs. The intensity was reported as number of egg/10 ml urine. The degree of intensity was categorized as light infection (≤ 50 ova/10 ml of urine) and as heavy infection (>50 ova/10 ml urine) according to the World Health Organization [11]. To rule out *Schistosoma mansoni* eggs and other intestinal helminths, stool specimens were collected from all individual who have *Schistosoma haematobium* eggs in their urine, and processed following the Kato katz procedure [8]. The urine samples of the participants were aliquoted in cryotubes and stored at -20.

Blood for cytokines assay

Five millilitres of venous blood was collected in plain container from each *Schistosoma haematobium* infected volunteer and allowed to clot at room temperature. The sera were separated using centrifugation at 3000 rpm for 10 minutes. Then aliquoted in cryotubes and frozen at -20°C. Their peripheral blood from all participant was examined for plasmodium using ICT for malaria with two species device (P.F and P.v) (SD Bio standard Diagnostics PVT-LTD India), to exclude malaria infection. All the participants were offered anti-helminthic treatment with the recommended dose of praziquantel, 40 mg/kg body weight after collection of blood samples. Malaria cases were treated according to the treatment regime prescribed by the Ministry of health in Sudan.

A second blood and urine sample was collected from all the participant four weeks after treatment and processed as before. All the samples were transported frozen in cold box to the laboratory of Parasitology Department in the College of Medical laboratory Sciences National Ribat University, and stored at -80°C. The samples were defrosted for the first time for prior to testing.

Control samples

A group of 42 individuals were chosen from the area of the study on the basis that they had negative urine samples for *Schistosoma haematobium* ova and had no past history of *Schistosoma*. Infection in addition they were neither ill nor under any type of therapy at the time of sample collection. This represent controls from the endemic area. Another group of 28 apparently healthy individuals, living in non-endemic area were included in the study as controls from a non-endemic area.

Cytokines measurement

Enzyme - linked immunosorbent assay (ELISA) kits e Bioscience were used to determine the levels of two cytokines (IFN γ and IL-2) in serum and urine samples of *Schistosoma haematobium* infected individuals and controls from the endemic and non-endemic areas. All samples were run in single and the concentrations were calculated using standard curves.

Assay procedures

The assay procedure is the same for two cytokines both in sera urine (Manufacture instruction). Ninety six polystyrene micro titer plate (corning costar 9018) were coated with recommended concentration of capture antibody 100 µl/well in coating buffer. The plate was sealed and incubated overnight at 4°C. Wells were aspirated (emptied) and washed five times with 250 µl/well washing buffer (one minute a time) was allowed for soaking during each wash step. Plate was blotted on absorbent paper. 200 µl of 1x assay diluents were added/well and the plate was incubated at room temperature for one hour (blocking). Aspiration and wash was done as before. Standards were diluted with 1x assay diluents. Two fold serial dilution of the top standards were performed to make the standard curve, and 100 µl/well of standard were added to appropriate wells, also 100 µl/well of samples were added to appropriate wells. The plate was sealed and incubated overnight at 4°C. Aspiration/wash was done as before. 100 µl/well of detection antibody diluted in 1 x assay diluents was added to each well, and the plate was sealed and incubated at room temperature for one hour. Aspiration and washing was done as before. Subsequently 100 µl of Avidin-horseradish peroxidase diluted in assay diluents was added to the wells and sealed and incubated at room temperature for 30 minutes. Aspiration/wash was done as before (soaked in wash buffer for 1-2 minutes prior to aspiration, repeated for a total of 7 washes. 100 µl/well of substrate solution was added to each well and then incubated at room temperature for 15 minute. The reaction were stopped by adding 50 µl of stop solution. The absorbance was measured with a microplate reader at 450 nm.

Statistical analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS version, 17). Thus parametric methods were used to evaluate results. The study population were categorized as infected (positive egg) or uninfected (endemic and non-endemic controls). Significance of difference, between mean values were assessed by student's t-test. P-values less than 0.05 were regarded as significant. The comparison between *Schistosoma* egg positive and egg negative were analyzed by chi-square test. The relation between cytokine levels and infection intensity, as well as between cytokines in pair were conducted using a one-tailed non parametric spearman correlation procedure [12].

Results

A total of 130 infected patients with *Schistosoma haematobium* were included in the study, 81 (62%) males and 49 (38%) females. The highest rate of infection were found among young people aged less 13 years (Table 1), Mean infection intensity for the study population was 61.92 eggs /10 ml urine, standard deviation 57.66. Figure 1 showed the intensity of the infection (53.1% light infection and 46.9% heavy infection), according to the WHO classification.

Age groups	Frequency of infection	Percent (%)
less 13	96	73.85
13-23	32	24.61
>23	2	1.54
Total	130	100

Table 1: Frequency of infection in different age groups of Sudanese patients infected with *Schistosoma haematobium*.

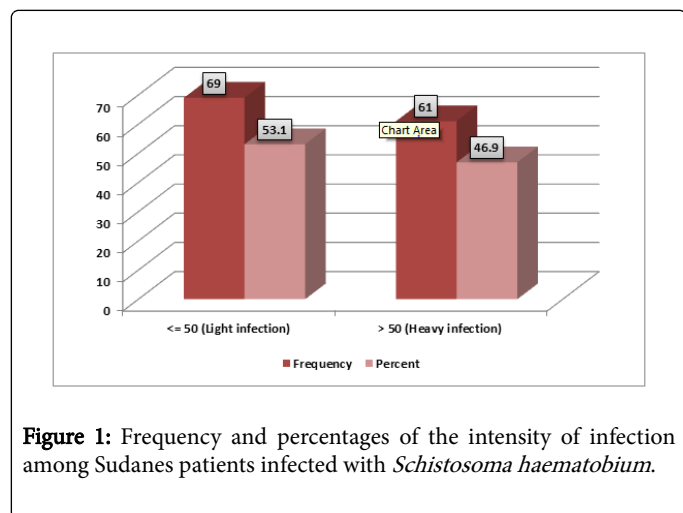


Figure 1: Frequency and percentages of the intensity of infection among Sudanese patients infected with *Schistosoma haematobium*.

Cytokine Profile

The cytokine profile measurement in sera of the infected individual, before and one month after treatment, showed high levels of IFN γ before treatment, but the difference was not significant (P=0.215) (Figure 2a). IL-2 was found to be high before treatment than after treatment. The difference before and after treatment was highly significant (P=0.007) (Figure 2b).

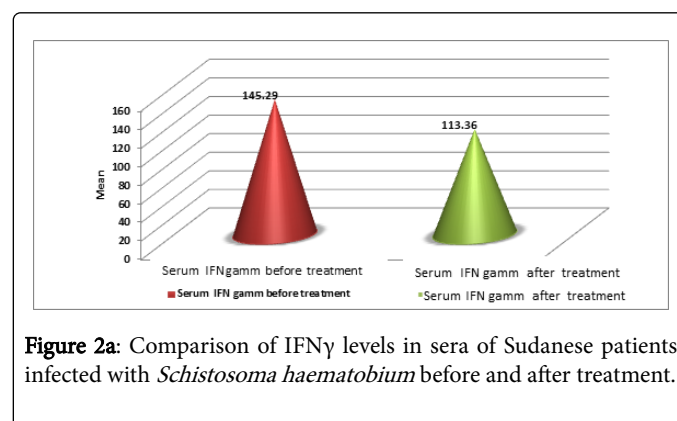


Figure 2a: Comparison of IFN γ levels in sera of Sudanese patients infected with *Schistosoma haematobium* before and after treatment.

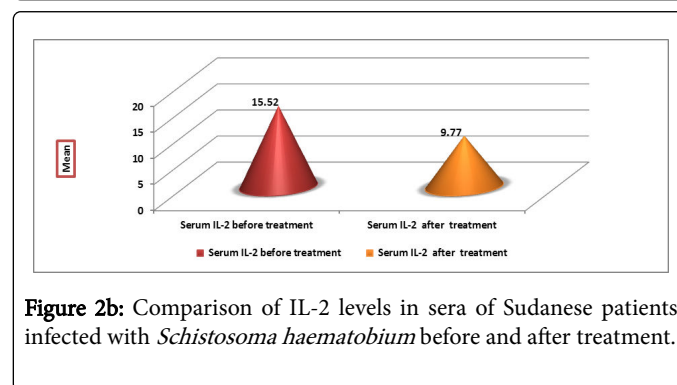


Figure 2b: Comparison of IL-2 levels in sera of Sudanese patients infected with *Schistosoma haematobium* before and after treatment.

	Cytokine	Mean	N	Standard deviation	P-value
Pair 1	IFN γ in urine before treatment	86.17	106	159.26	0.000
	IFN γ in urine after treatment	21.778	106	56.22	
Pair 2	IL-2 in urine before treatment	38.76	104	76.59	0.000
	IL-2 in urine after treatment	7.04	104	21.89	

Table 2: Cytokine profile in the urine of Sudanese patients infected with *Schistosoma haematobium* before and after treatment.

Table 2 summaries the cytokine profile in urine before treatment and one month after treatment. There were high levels of different cytokines before treatment than after treatment. The differences were statistically significant for IFN γ (P=0.000), IL-2 (P=0.000). The levels of IFN γ and IL-2 detected in serum and urine showed the same pattern, high before treatment and low after treatment.

Table 3 summarize the serum level of cytokines detected in different groups (infected individual before and after treatment, controls from

endemic and non-endemic area). IFN γ level in infected samples before and after treatment was 140.15 ± 232.63 and 112 ± 215.75 respectively, in controls from endemic area was 70.21 ± 96.66 and in controls from non-endemic area was 8.13 ± 10.31 . There were differences in the levels of the IFN γ detected after treatment. IL-2 in infected samples was 15.42 ± 30.45 before treatment and 9.77 ± 18.52 after treatment. In control from endemic and non-endemic area IL-2 levels were 7.64 ± 17.76 and 3.12 ± 0.59 respectively. The highest levels of the tested cytokines detected in the three groups is IFN γ .

Samples group	Mean	Before treatment		After treatment	
		Std	Mean	Std	Mean
Infected	140.15	232.63	112.52	215.75	
Control from endemic	70.21	97.66	NA	NA	
Control from non-endemic	8.13	10.31	NA	NA	
Infected	15.42	30.45	9.77	18.52	
Control from endemic	7.64	17.76	NA	NA	
Control from non-endemic	3.12	0.59	NA	NA	

Table 3: Mean and standard deviation of the levels of cytokines in the sera of infected individuals before and after treatment and in the sera of controls groups.

Dependent Variable	Sample Groups	Sample Groups	Sig.
IFN γ in serum before treatment	Infected	Control from endemic	0.045*
		Control from non-endemic	0.005*
	Control from endemic	Infected	0.045*
		Control from non-endemic	0.237
	Control from non-endemic	Infected	0.005*
		Control from endemic	0.237
IL-2 in serum before treatment	Infected	Control from endemic	0.095
		Control from non-endemic	0.049*
	Control from endemic	Infected	0.095
		Control from non-endemic	0.52
	Control from non-endemic	Infected	0.049
		Control from endemic	0.52

Table 4: Comparisons of cytokines (IFN γ and IL-2) in the sera of different sample groups. *The mean difference is significant at the 0.05 level.

Table 4 summarize the cytokine level in the different study groups are summarized as follows: comparison of the level of IFN γ in infected

Cytokines	Groups	Mean \pm SD	T-test	P-value
INF γ	Light infected group (n=69)	97.71 \pm 126.69	82.19	0.00069*
	Heavy infected group (n=61)	187.13 \pm 304.98		
IL-2	Light infected group (n=69)	13.93 \pm 28.98	11.64	0.0129*
	Heavy infected group (n=61)	17.12 \pm 32.19		
	Heavy infected group (n=61)	33.08 \pm 44.26		

Table 7: Relation between intensity of infection and cytokine levels in the sera of Sudanese patients infected with *S.haematobium*.

samples with the control samples from endemic and non-endemic area was found to be significant (P=0.045 and 0.005) respectively. When IFN γ level compared in endemic controls with the non-endemic one it was not significant (P=0.237). The comparison of IL-2 in infected samples with endemic controls was not significant (P=0.095) and the infected samples with non-endemic controls was significant (P=0.049) while the comparison of the two controls was not significant (P=0.520).

Cytokines	Sex	Mean \pm STD	T-test	P-value
INF γ	Male (n=81)	115.98 \pm 16.14	76.03	0.0085**
	Female (n=49)	178.82 \pm 314.87		
IL-2	Male (n=81)	14.01 \pm 29.72	24.91	0.0077**
	Female (n=49)	17.76 \pm 31.78		

Table 5: Comparison of cytokine profile in sera of Sudanese males and females infected with *S.haematobium* before treatment.

Table 5 shows sex cytokine profiles in patients before treatment. Females produce high levels of IFN γ and IL-2, than the males and there were significant differences (P=0.0085, P=0.0077 respectively).

Cytokine	Age	Mean \pm SD	Comparison	T-test	P-value
INF γ	<13	155.28 \pm 254.28	(<13) vs. (13-23)	38.41	0.0083**
	13-23	67.23 \pm 88.81	(<13) vs. (>23)	56.29	0.0059**
	>23	505.258 \pm 184.34	(13-23) vs. (>23)	73.16	0.0017**
IL-2	<13	15.44 \pm 32.29	(<13) vs. (13-23)	3.28	0.0628NS
	13-23	13.83 \pm 24.67	(<13) vs. (>23)	14.56	0.0124*
	>23	39.45 \pm 1.84	(13-23) vs. (>23)	12.79	0.0235*

*P-value \leq 0.05 is considered significant. **P-value \leq 0.01 highly significant NS is non-significant

Table 6: Age cytokine profile in sera of infected groups before treatment.

The cytokine profiles in the different age groups before treatment are shown in Table 6. The levels of both cytokines (IFN γ and IL-2) appear to be influenced by age. High levels were detected in those >23 year of age. The differences in cytokine levels in different age groups were significant in case of IFN γ . But IL-2, shows no significant differences in age groups (<13) vs. (13-23) (P=0.0628).

The relation between the intensity of the infection and cytokine production appear in table (7). Both cytokines (IFN γ and IL-2) were found to be high in heavy infected individuals and the differences were statistically significant (P=0.00069, P=0.0129, respectively).

Cytokines	Groups	Mean \pm SD	T-test	P-value
IFN γ	Light infected group (n=69)	111.20 \pm 193.13	58.29	0.00092**
	Heavy infected group (n=61)	62.59 \pm 123.67		
IL-2	Light infected group (n=69)	40.22 \pm 84.95	17.41	0.0082'
	Heavy infected group (n=61)	34.10 \pm 75.81		

Table 8: Relation between intensity of infection and cytokine levels in the urine of Sudanese patients infected with *S.haematobium*.

The level of cytokines in urine in relation to the intensity of infection were found to be as follows. IFN γ shows high levels in both heavy and light infection with significant differences (P=0.00069). IL-2 levels were relatively low in both high and low infection (Table 8).

Discussion

The relationship between age, intensity of infection and gender has been observed in many epidemiological situations, endemic and epidemic [6,13].

This study aimed to determine the relation between urinary and serum cytokine level in Sudanese individual infected with *Schistosoma haematobium*, which were measured before and after treatment. The cytokines studied were IFN γ , and IL-2 (markers of Th1 responses). All participants had a detectable levels of baths cytokines with different concentration. The most dominant cytokine was found to be IFN γ , that raised early in the infection with *Schistosoma*. Although it has been proposed that elevated levels of IFN γ may be associated with susceptibility to *Schistosoma* infection, however findings in this study suggest that IFN γ may be involved in early stages of the immune response to *Schistosoma* infection and this is in agreement with finding reported by Mutapi et al. and Mduluza et al. [14,15]. The IFN γ and IL-2 cytokines which observed in the study may indicate a regulatory role of Th1 cytokine in early responses to infection and this finding concur with Arnaud V et al. [16]. Also the study showed there was a decrease in IFN γ and IL-2 after treatment and this is on line with the Mutapi F et al. finding [14]. The Th1 cytokine IFN γ was found to exist during infection but the cytokine persisted at lower levels in treated individual and this may be use to modulating effect of IL-10. Th1 responses have been linked to the so called endemic normal [17,18]. When uninfected healthy individuals living in an area, where *Schistosoma* is transmitted, produce high levels of parasite specific IFN γ and low levels of IL-10. This coincide with our finding of these cytokine among endemic control individuals. This study also showed high levels of cytokines in females than in males. Similar observation were seen in immunoglobulin's in other studies. This could be due to hormonal influence. We also observed in this study an association between age and cytokines levels. Furthermore this study showed a positive correlation between age and cytokine as evidenced by the increase of IFN γ with age and the decrease of IL-2 with aging. This finding is comparable with report of Milner et al. regarding IFN γ , but not IL-2. The cytokine levels in urine showed similar results as

observed in serum, high before treatment and the levels were low after treatment. Some cytokines were detected with high concentration in urine than in sera which may be due to local production in the bladder. These finding concur with those reported by Gurgoze et al. who studied cytokines in serum and urine of children infected with urinary bacteria [19]. Very recently a similar finding was reported by Kariuki et al. who studied urinary cytokine and related it to urinary tract pathology in children [7].

Conclusion

Depending on high production of IFN γ and IL-2 and the low production of IL-4 and IL-5, the immunological classification of urinary Schistosomiasis in the study area could be considered in the acute stage of the disease. Also the result showed that IL-10 responses develop early compared to IL-5 and IL-4 responses and may be down modulating immune pathological responses that occur during the early phase of infection. IL-4 and IL-5 were working in reverse to IL-10 when studying these in sera and urine before and after treatment and we deduce that it was due to the modulating effect of IL-10. The study also showed that it is possible to use urine to study different cytokines in urinary Schistosomiasis which may indicate that there is a possibility of local production of cytokines in bladder.

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