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Serum Levels of Interferon-gamma in Patients with Falciparum Malaria- Khartoum State- Sudan

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Abstract: *A complex parasite such as human Plasmodium is likely to generate a variety of substances that injure the hosts directly or cause immunopathology. In malaria, a blood concentration of anti-inflammatory cytokines, such as interferon-gamma (IFN-γ) is increased. The present study was performed to analyze IFN-γ levels in patients with certain conditions of malaria with healthy controls and correlate with malaria density infection as well as age groups. It is a cross-sectional study was carried out in Khartoum State/ Sudan, a total of 49 febrile patients and 10 healthy volunteers as controls were included in this study. Blood samples were collected from both patients and control candidates in (EDTA) containers for parasitological and immunological tests. Microscopical examination of thick and thin blood films rather than immunochromatography test (ICT) were used to detect the positive samples for Plasmodia. Enzyme linked Immunosorbant Assay (ELISA) was used for the determination of IFN-γ levels. The results showed that the prevalence was occurred among all age groups and P.falciparum was the predominant species with P.vivax mono or co-infections. Furthermore IFN-γ levels in malaria patients were 65.14and SD 64.56 while the levels in healthy participants were 12.33 and SD was 4.11. The difference was found to be statistically significant (P= 0.001). The levels of cytokine mentioned above were observed to be raised in malaria individual compared to healthy control candidates.*

Keywords— *P.falciparum*; *P.vivax*; Giemsa; Immunochromatographic; IFN-γ; Khartoum State

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1. INTRODUCTION

Malaria is widespread in tropical and subtropical regions, there are approximately 350-500 million cases, with 1 to 3 million morbidity, the majority among young children in sub-Saharan Africa [1], about 90% of them in Africa and about half of the world's population (3.3 billion people) is at risk of malaria in more than 100 countries. It's spreading as a result of environmental changes and drug resistance [2]. Malaria is commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development [3]. Species of medical importance are; the most serious forms are caused by *Plasmodium falciparum.* Malaria caused by *P.vivax, P.ovale* and *P.malariae* causes milder disease that is not generally fatal. A fifth species, *P.knowlesi*, causes malaria in macaques but can also infect humans. [4,5]. The clinical manifestation ranging from asymptomatic, uncomplicated to life-threading complications which common found in sub-Saharan Africa [6]. Sudan is considered as a high burden of malaria-related morbidity and mortality. However, with WHO's support to the national malaria control programme, morbidity and mortality has reduced the cases from more than four million in 2000 to less than one million in 2010. Between 2001 and 2010, the number of deaths due to malaria reduced by 75%. WHO works in close collaboration with the national malaria control programme to implement appropriate and cost-effective malaria control interventions [7]. The pathogenesis of malaria is due to massive hemolysis of infected cell rather than sensitized uninfected cell induced by hemozoin and malarial pigment increasing the severity of anaemia. Cytoadherence in falciparum is due knobes expressed on parasitized red cell and specific receptor complex on the endothelial cells cause the most severe complications [8]. The criteria and mechanism of severity in *P. vivax* may differ from falciparum malaria and not related to parasite biomass. There is a 4- to 5-fold greater loss of uninfected red cells in *P.vivax* relative to *P. falciparum* infection at low parasite densities (<2%) that produce severe anaemia or other complications [9]. Protective anti-malarial immunity reflects as antibody production, phagocytosis, cellular cytotoxicity and parasite inhibition exerted by lymphocytes, neutrophils and mononuclear phagocytes. However, some of these cellular activities may also cause tissue damage and the course of a malaria infection is highly dependent on the balance between the cytokines secreted by the various cells when activated [10]. In any event, proinflammatory cytokines such as IFN γ, IL-1, IL-6 and others may be protective by inducing parasite killing by monocytes/macrophages and neutrophils. IL-12, produced by mononuclear phagocytes and

other cells, contributes to protection against pre-erythrocytic and blood infection by initiating a Th1 anti-malaria response in mice as well as in monkeys [11].

2. MATERIALS AND METHODS

2.1 Study design:
This is a cross-sectional study.

2.2 Study area and study period:
The study was conducted in Medical Diagnostic Center-Khartoum State- Sudan during the period from December 2016 to December 2018.

2.3 Study population:
The study was carried out on patients that clinically diagnosed with malaria in addition of ten volunteers as control.

2.4 Inclusion criteria:
Patients with history of fever proceeding in the past 24 hours with confirmed malaria parasite positive slides and ICT were included study participant's cases and healthy volunteers were included as the study controls.

2.5 Exclusion criteria:
All patients who were negatively diagnosed for malaria parasites. Patients with any known concurrent chronic illness and diseases such as malnutrition, chronic infection as tuberculosis, pneumonia or urethritis in addition to known cancer patients.

2.6 Sample size:
The sample size was obtained according to the statistical method for determining sample size as following equation as described by Mendenhall *et al*. (1981) [12].

$$N = t^2 * P\ (1-p)/M^2)$$

N= Sample size

t = The normal standard deviate (t = 1.96)

P = The frequency of occurrence of malaria (16%)

M= Degree of precision (0.05%)

According to the above equation, the study was conducted among 200 clinically suspected individuals. Only 49 patients whom were diagnosed with positive malaria were included in the study. In addition to 10 volunteers were included as control.

3. METHODS

3.1 Sample collection and processing:
Fifty nine blood samples were collected from all participants. The blood was drawn aseptically into EDTA Vacutainer® tubes (Becton Dickson and company, Franklin Lakes, NJ, USA). From each specimen had been tested by the both methods as dry films and Immune Chromatographic Test (ICT), then vacutainers were centrifuged at 1,200 g for 10 minutes at room temperature. The plasma was out and the samples were aliquoted and stored at -20°C until assayed. All questionnaires were filled by participants [13,14].

3.2 Preparation of thick film:
After collection of blood on a clean and grease free glass slide, thick film was made by spreading one drop of blood with a spreader evenly on an area about 15×15 mm in diameter. Care was taken to avoid rouleaux formation. Then, the slide was labeled properly and allowed to air-dry by keeping the slide on horizontal position. Precaution was taken during spreading and drying [13].

3.3 Preparation of thin film:
After collection of one drop of blood on a clean grease free slide, thin film was made by spreading the blood using a smooth edged slide or spreader at an angle of 45º from the horizontal plane. A well-prepared thin blood film was judged by having a smooth tail end and free of vertical lines and holes. The slide was then labeled properly and allowed to air-dry [13]. Absolute methanol or ethanol was used to fix the thin film. Following steps were taken for fixing the thin film as described by Cheeshbrough (1999) [13]:

- The slide was placed horizontally on a staining rack.
- A small drop of absolute methanol or ethanol was applied to the thin film.
- Then the slide was allowed to fix for 1-2 minutes.

Staining of the films:

The slide was first placed on a staining rack. Then 10% Giemsa stain having a pH of 7.2 was poured gently on the fixed thin film or de-hemoglobinized thick film until the slide was totally covered. Then the slide was allowed to stain for 30-45 minutes out of the sunlight. Then the stain was washed with clean water. Back of the slide was wiped and placed in a draining rack. The slide was then allowed for air-dry [13].

3.4 Microscopic examination of the stained film
Thick film:

The thick film was examined first by using the 40x objective, a well-stained part of the thick film was selected which was well populated with WBC. Then the selected portion of the film was examined with 100x oil immersion objective by moving along the width of the slide. At least 100 fields were examined before a slide was considered as negative for malaria parasite [13].

Thin film:

The thin film was examined by the 40x objective first followed by 100 x oil immersion objectives. After applying immersion oil the film was examined by moving along the edge of the film. Then moving the slide inward by one field, returning in a lateral movement and so on. At

least 100 fields were examined before a slide was considered as negative for malaria parasite [13].

3.5 Determination of malaria parasitaemia:

Parasites density determination:

Semi quantitative count (thick film) was used the following semi-quantitative scale:

+ 1-10 asexual parasites per 100 thick film fields
++ 11-100 asexual parasites per 100 thick film fields
+++ 1-10 asexual parasites per single thick film field
++++ More than 10 asexual parasites per single thick film field [10]

3.6 Detection of Antigen:

Antigen was detected by immunochromatographic (ICT) method with malaria *P.falciparum* and *P.vivax Plasmodium.* Antigen Onsite Rapid Screening Kit. The kit was manufactured by Onsite malaria P.f/P.v Ag Rapid test, CTK Biotech. Inc. USA. Lot no: F0323N9102. Test procedure was performed according to manufactories instructions.

Principle of the test:

The malaria onsite Rapid test is one step chromatograhic immunoassay which specifically detects the Antigens of *P. falciparum and Plasmodium.vivax* in human whole blood. The test utilizes *P.falciparum* specific antigen HRP-II and pLHD *P. vivax*-specific Antigen to ensure test specificity and sensitivity.

3.7 Cytokine assays

The serum levels of the IFN- γ was evaluated by sandwich enzyme-linked immunosorbent assays (ELISA), using pairs of cytokine-specific monoclonal antibodies provided by the commercially available test (BioLegend's ELISA MAX™ Deluxe Sets Cat. No. 430104, San Diego, California USA). All tests were performed according to the manufacturer's instructions. Plate included a standard curve of recombinant human cytokine run in parallel with the samples. All samples were measured and the optical density was used for all analyses.

3.9 Ethical consideration and permission:

Ethical approval was sought from the Sudan University of Science and Technology, in addition to an individual orientation for patients about the purpose and benefit of the study were explained.

3.10 Data analysis:

Data were analyzed using Statistical Package for Social Sciences (SPSS) under windows, version 16.0. Pearson Chi-Square statistical analysis was performed and the *p* values of less than 0.05 were considered statistically significant. In addition to **ANOVA** test is also used.

3.11 Sensitivity of ICT techniques:

Sensitivity was calculated as described by Kocharekar *et al.* [15]:

$$TP/(TP+FN) \times 100\%$$

TP= True positive

FN= False negative

4. RESULTS

The study was conducted on 59 participant 49 (83.1%) were patients and 10 (16.9) were volunteers, 22 (37.3%) were males and 37 (62.7%) were females in (Table1). The age between 1-70 years old, the mean age was 30±17 years old. Study participants were divided into 7 age groups as follow: less than 10, 11-20, 21-30, 31- 40, 41-50, 51-60 and more than 61 years old, the frequency of each age group was 8 (13.6%), 11 (18.6%), 16 (27.1%), 10 (16.9%), 6 (10.2), 6 (10.2) and 2 (3.4%) respectively in (Table2) (Figure1).

Table 1: The frequency of gender

Gender	Frequency	Percent
Male	22	37.3
Female	37	62.7
Total	**59**	**100.0**

Table 2: The frequency of age groups

Age group	Frequency	Percent
Less than 10	8	13.6
11-20	11	18.6
21-30	16	27.1
31-40	10	16.9
41-50	6	10.2
51-60	6	10.2
More than 61	2	3.4
Total	**59**	**100.0**

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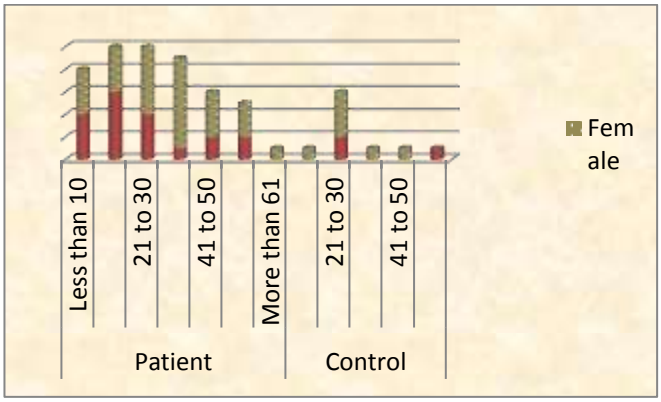

Figure 1 : the frequency of gender among the age groups in both patient and control

Among the 59 individuals the Giemsa stained blood films showed 10 (16.9%) were negative as control, while 49 (83.1%) positive with different parasitemia as 39 (66.1%) were positive for *P.falciparum* and 8 (13.6%) were positive for *P.*vivax and only 2 (3.4%) were mixed infections (Tables 3) (Figure 2). ICT showed sensitivity of 91.8% among the individuals as 38 (64.4%), 5 (8.5%), 2 (3.4%) and 14 (23.7%) respectively (Tables 4) (Figure 3). The correlation between blood film and ICT results among species were statistically significant at *P*=0.000.

Table 3: The results of blood films among the individuals

Species	Parasitemia				Total
	Zero	Low	Moderate	High	
Negative	10	0	0	0	10
P.falciparum	0	25	12	2	39
P.vivax	0	4	2	2	8
Mixed *P.f*&*P.v*	0	1	1	0	2
Total	**10**	**30**	**15**	**4**	**59**

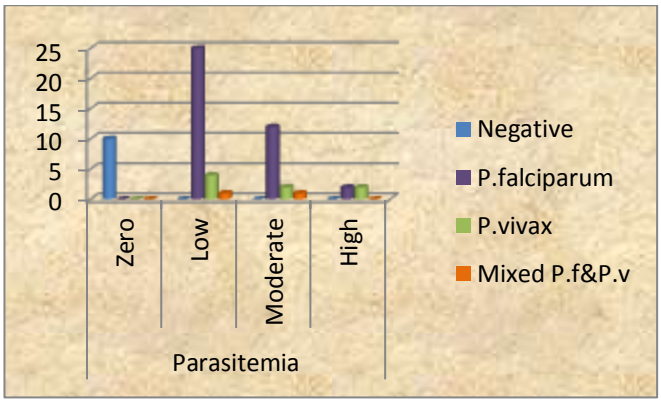

Figure 2 : The reslut of Giemsa stained blood films and parasitemia

Table 4: The results of blood films and ICT.

Species	B.F.F.M		ICT		*P*.value
	No	%	No	%	
Negative	10	16.9	14	23.7	0.000
P.falciparum	39	66.1	38	64.4	
P.vivax	8	13.6	5	8.5	
Mixed *P.f* & *P.v*	2	3.4	2	3.4	
Total	59	100.0	59	100.0	

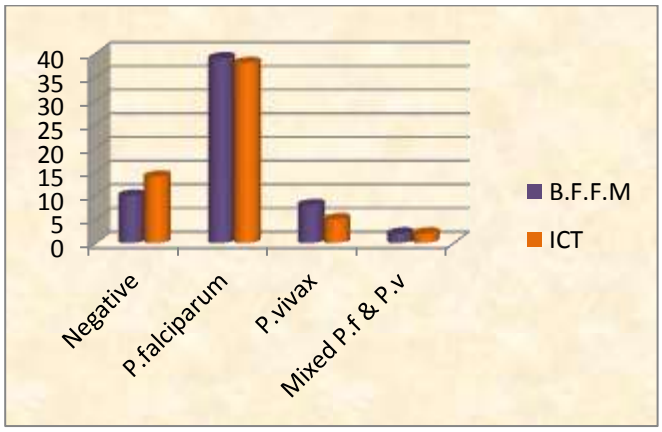

Figure 3 : Comparison between results of boold films and ICT

Concerning the gender and age groups, the results showed that the difference was found to be statistically insignificant at *P*=0.943 and *P*=0.086 respectively.

The results showed that IFN-γ levels in malaria patients was 65.14 and SD was 64.56, while the levels in healthy participants was 12.33 and SD was 4.11. The difference was found to be statistically significant (*P*= 0.0001) (Table 5). Moreover, mean of IFN-γ showed that there was significant statistical difference at the 0.05 level with the species specially in low paraitemia (Table 6) (Figure 4), but there was no significant difference concerning the age groups (0.931) regarding mean of IFN-γ level. The results were showed insignificant correlation (0.065) between the IFN-γ and species .

Table 5: Mean and SD of IFN-γ in patients and control

Group Sample		IFN-γ (ng/ml)	*P*. value
Patients	**Mean**	65.14	0.0001
	SD	64.56	
Control	**Mean**	12.33	
	SD	4.11	

Table 6: Mean and SD of IFN-γ in patients and control

Parasitemia	Mean	S. D	Sig
Zero	12.33	4.11	0.05
Low	74.54	71.04	
Moderate	55.25	56.56	
High	31.78	10.55	

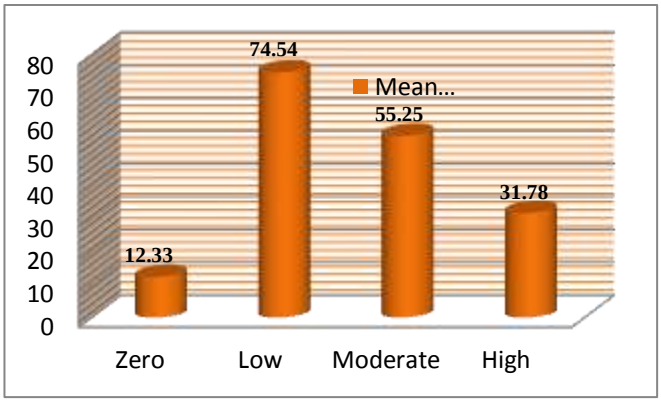

Figure 4: Mean of IFN-γ level among different parasitemia

5. DISCUSSION

Malaria infection gives rise to host responses which are regulated by both the innate and adaptive immune system as well as by environmental factors. Acquired immunity is both species- and stage-specific that initiated by pro-inflammatory cytokines such as IFN γ, IL-1, IL-6 and other cytokines which may be protective by inducing parasite killing by phagocytes and neutrophils [16]. However, cytokines had crutial roles in both the clinical effects due to pathogenesis of malaria in addition to the strong cellular immune response mainly due to the pro-inflammatory cytokines IL-12 and IFN-γ [9].

This study aimed to determine the correlation between IFN-γ levels cytokine and malaria infection. It is thought that many factors affect their levels.

The pro-inflammatory cytokines, IFN-γ levels were elevated in patients with malaria compared to healthy controls; patients with low parasitemia had much greater elevated levels of IFN-γ which initiated by early T helper type-1 response compared to the other patients with higher parasitemia [17], while in higher parasitemia the regulatory role of T-helper type 2 responses activated the production of anti-inflammatory cytokines were required to protect against excessive release of IFN-γ to determine the beneficial effects on the host's ability to cope with infection and combat disease [11].

Consistent with the present results, study carried by Artavanis *et al.* (2002), showed that rapid and intense IFN-γ response from malaria-naive PBMC than do *P. falciparum* schizont lysates correlating with rapid iRBC activation of the NK cell population to produce IFN-γ [17]. In the same way this study showed that there was no correlation between IFN-γ level and the age of infected patients because the study area considered as non-endemic malaria transmission and this result was compatible with Prakash *et al.* (2006) study that showed IFN-γ and other cytokines as IL-2, IL-5, IL-6, and IL-12 levels were increased during infection predominantly in patients with mild malaria in comparison with severe malaria or complicated malaria regardless the age [18].

Experimental malaria studies performed exclusively in adult hosts indicated that both CD4+ and CD8+ T cells play an important role in the defense against malaria. To address the role of these cells in an age-dependent model, where young susceptible rats can be protected by the transfer of whole spleen cells from adult protected rats, TCR cells were transferred to young infected rats. These experiments indicated that 58% of young rats recovered from infection after T cell transfer [16].

Furthermore, RDTs designed to detect *P.falciparum* and *P. vivax* antigens as mixed infection, appear to be less accurate in malaria diagnosis compared to microscopy, these tests fail to detect around 8.2% of the cases and this percent was higher in compare of 4% failure as study done by Hamza *et al.* (2016) [19].

The results study showed that *P.falciparum* as the predominant species which was consistent with Hamza *et al.* (2016) study [19], but with presence of *P.vivax* infection rather than mixed *P.falciparum-P.vivax* infections in the study area.

6. CONCLUSION:

This study concluded that there was positive relation between IFN-γ levels and malaria infection among Sudanese patients related to paraitemia, showing that the levels of IFN-γ were increasing in low paraitemia. In addition to that *P.falciparum* as the predominant species in Khartoum.

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