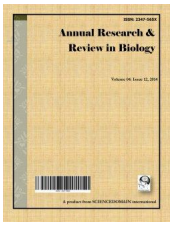

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Variations in Plasma IL-2 and IL-10 in Relationship with Plasmodium Parasite Density

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Authors' contributions

This work was carried out in collaboration between all authors. Author MFO designed the study, authors MFO, SGO and TA performed the statistical analysis, authors MFO, FRO, TA and SGO wrote the protocol, and wrote the first draft of the manuscript. Authors MFO, TA and FRO managed the analyses of the study. Authors MFO, FRO and SGO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Study Background: Plasma IL-2 and IL-10 are cytokines for immuno regulation and immuno modulation in infectious diseases. IL10 inhibit synthesis of IFN gamma, IL-2, IL-3, TNF. Plasmodium infection is associated with reproduction of cytokines for innate and acquired immunity. **Aim and Objective:** This work was designed to determine variations in plasma IL-2 and IL-10 in relationship with plasmodium parasite density.
Materials and Methods: Out of one hundred and sixteen (116) initially recruited only fifty Plasmodium infected female and male (female =25; male =25) aged 4-70 years free of *M. tuberculosis* and seronegative to HBsAg, HCV and HIV were recruited for the work.. Fifty age matched Plasmodium non-infected subjects were studied as control (female =25; male =25). Only subjects who were free of *M. tuberculosis* and seronegative to HIV, HCV, HBsAg test and AFB negative were recruited for the work. Plasma IL-2, IL-10, HIV, HBsAg and HCV were determined in the patients and the control subject immunochemically by ELISA while Identification of *Plasmodium spp* was determined in the blood of the patients and the control subject using WHO standard

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technique for the laboratory diagnosis of plasmodium infection in malaria endemic area.
Results: A frequency of : 12.0%(14) Anti-HCV seropositive, 6.9%(8) Anti-HIV seropositive, 19.8%(23) HBsAg seropositive, 13.8% (16) AFB positive patients and 4.3%(5) indeterminate results was obtained from the 116 Plasmodium infected patients initially recruited. There was a significantly Higher plasma value of IL-10 in plasmodium infected patients with parasite density of 500-999 and ≥1000 than the control subjects with $p<0.05$. There was also a significantly lower mean plasma value of IL-10 in plasmodium infected patients with parasite density of 50-499 than those patients with parasite density 500-999 and ≥1000 with $p<0.05$.
Conclusion: Plasmodium parasitemia and increase in parasite density has been found to significantly increase the plasma value of IL-10 with no significant change in the plasma value of IL-2. There was also an evidence of HIV, HCV, HBV and *M. tuberculosis* co-infection with *Plasmodium spp* .

Keywords: Variations; IL-2; IL-10; Plasmodium parasite density; anti-HCV; anti-HIV; HBsAg; AFB.

1. INTRODUCTION

Plasmodium infection results into malaria fever. The severity of the infection varies with parasite density. The IL-2 and IL-10are immune material and examples of cytokines especially interleukins. Interleukins stimulates both cellular and humeral responses. They are also involved in innate and acquired immunity. Plasmodia species that infect human and are of medical importance include those of vivax, falciparum, ovale and maleriae. The pathophysiology of plasmodium infection involves destruction of red blood cells and stimulation of the production of cytokines which may in turn stimulate the production of specific antibodies to the infecting specie of plasmodium [1]

The role of interleukin-10 and interleukin-2 (IL-10 and IL-2) in malaria are poorly characterized. IL-10 polymorphisms and IL-10 production capacity were associated with clinical malaria infections in young children. High IL-10 production capacity inherited from parents may diminish immunological protection against *P. falciparum* infection, thereby being a risk for increased malaria morbidity [1]

There is an existing Immunological interaction between IL-10 and IL-2 in infection, IL-10 inhibits human T cell proliferation and IL-2 production in response to mitogen. Inhibition of IFN-gamma production by IL-10 appears to be independent of the cytokine effect of IL-2 production [2] IL-10 and IL-2 synergistically enhance Ig production of SA-activated B cells in a mechanism which is different from the upregulation of IL-2 receptors. Moreover, the data emphasize the importance of the interplay of IL-2 and IL-10 in determining the outcome of humoral immune responses [3]

IL-2 T lymphocytes regulate the growth and differentiation of T cells and certain B cells. IL-2 also stimulates some B cells for the production of antibodies in infections [3]. Interleukin-2 (IL-2) regulates the activities of white blood cells (often lymphocytes) responsible for immunity. IL-2 is part of the natural immune response to microbial infection in the body system and it helps the body to discriminate between foreign ("non-self") and "self". IL-2 binds to IL-2 receptors, which are expressed by lymphocytes to mediate its effects [4,5].Interleukin 10 (IL-10 otherwise known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine that enhances the survival of B cell ,proliferation, and antibody production [6-10].

There is little information on the interplay between IL-2 and IL-10 in plasmodium infection hence the justification for this this work. This work aimed at determining the possible variations in plasma IL-2 and Il-10 in relationship with plasmodium parasite density.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Study area

The study was carried out in Saki West at Baptist Medical Centre Saki-Nigeria. Saki West is a Local Government Area in Oyo State, Nigeria. Its headquarters are in the town of Saki. It has an area of 2,014 km² and a population of 278,002 at the 2006 census. Shaki,Nigeria is located at the extreme end of Oyo state. It has a Ressetlement center of 2nd Mechanised division of Nigerian Army, Baptist School of Nursing and Midwifery, School of Medical Laboratory Technology, Baptist Medical Centre, Saki, School of Basic

Midwifery, Muslim Hospital, Saki, Baptist Medical Centre, Saki, State Hospital, Saki, Muslim Hospital, Saki, The Oke-Ogun Polytechnic, and a Technical college. Shaki,Nigeria is also one of the largest city in Oyo state. The postal code of the area is 203.

2.1.2 Study population

Out of one hundred and sixteen (116) initially recruited only fifty Plasmodium infected female and male (female =25; male =25) aged 4-70years free of *M. tuberculosis* and seronegative to HBsAg, HCV and HIV were recruited from the medical out-patient Department of Baptist Medical Centre Saki-Nigeria. Fifty age matched Plasmodium non-infected subjects were studied as control (female =25; male =25). Only subjects who were free of *M. tuberculosis* and seronegative to HIV, HCV, HBsAg test and AFB negative were recruited for the work.

2.1.3 Biological sample

Five milliliter of venous blood was obtained from each of the subjects and preserved inn lithium Heparinized anti-coagulated bottles for the identification of plasmodium and determination of IL-10 and IL-2.

2.2 Methods

2.2.1 Plasma IL-2 ELISA assay

Plasma IL-2 Assay was carried out using Abcam's IL-2 (Interleukin-2) Human ELISA (Enzyme-Linked Immunosorbent Assay) kit. It is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of Human IL-2 in plasma, and cell culture supernatants. (Human IL-2 concentration is pretty low in normal plasma, it may not be detected in this assay).This assay employs an antibody specific for Human IL-2 coated on a 96-well plate. Standards and samples are pipetted into the wells and IL-2 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Human IL-2 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of IL-2 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2.2.2 Plasma IL-10 ELISA assay

Plasma IL-10 was carried out using Abcam's kit. Abcam's IL-10 (Interleukin-10) Human *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of IL-10 in supernatants, buffered soloutions, serum and plasma samples.

A monoclonal antibody specific for IL-10 has been coated onto the wells of the microtiter strips provided. Samples, including standards of known IL-10 concentrations, control specimens or unknowns are pipetted into these wells. During the first incubation, the standards or samples and a biotinylated monoclonal antibody specific for IL-10 are simultaneously incubated. After washing, the enzyme Streptavidin-HRP, that binds the biotinylated antibody is added, incubated and washed. A TMB substrate solution is added which acts on the bound enzyme to induce a colored reaction product. The intensity of this colored product is directly proportional to the concentration of IL-10 present in the samples.

2.2.3 Anti HCV ELISA assay

This was carried out using Anti-Hepatitis C Virus Core Antigen antibody (ab50288) Abcam kit. The hepatitis C virus (HCV) core protein represents the first 191 amino acids of the viral precursor polyprotein and is cotranslationally inserted into the membrane of the endoplasmic reticulum. Hepatitis C virus (HCV) core is a viral structural protein; it also participates in some cellular processes, including transcriptional regulation. However the mechanisms of core-mediated transcriptional regulation remain poorly understood. Hepatitis C virus (HCV) core protein is thought to contribute to HCV pathogenesis through its interaction with various signal transduction pathways. In addition, HCV core antigen is a recently developed marker of hepatitis C infection. The HCV core protein has been previously shown to circulate in the bloodstream of HCV-infected patients and inhibit host immunity through an interaction with gC1qR. Hepatitis C Virus is a positive, single stranded RNA virus in the Flaviviridae family. The genome is approximately 10,000 nucleotides and encodes a single polyprotein of about 3,000 amino acids. The polyprotein is processed by host cell and viral proteases into three major structural proteins and several non structural proteins necessary for viral replication. Hepatitis C virus (HCV) causes most cases of non-A, non-B hepatitis and results in most HCV infected

people developing chronic infections, liver cirrhosis and hepatocellular carcinoma. T cell responses, including interferon-gamma production are severely suppressed in chronic HCV patients.

2.2.4 HIV ELISA test

HIV test was carried out using Genscreen™ ULTRA HIV Ag-Ab Biorad Kit.

2.2.4.1Principle of the test

The Genscreen™ ULTRA HIV Ag-Ab is an enzyme immunoassay based on the principle of the sandwich technique for the detection of HIV antigen and of the various antibodies associated with HIV-1 and/or HIV-2 virus in human serum or plasma. The solid phase is coated with:

- monoclonal antibodies against p24 HIV-1 antigen
- purified antigens : gp160 recombinant protein, a synthetic peptide mimicking a totally artificial (i.e. encoded by no existing virus) HIV-1 group O-specific epitope and a peptide mimicking the immunodominant epitope of the HIV-2 envelope protein. The conjugates are based upon the use of:
- biotinylated polyclonal antibodies to HIV Ag (conjugate 1)
- Streptavidin and HIV antigens - peroxidase conjugate (gp41 and gp36 peptides mimicking the immunodominant epitopes of the HIV-1 and HIV-2 envelope glycoproteins, and the same synthetic peptide mimicking a totally artificial HIV-1 group O-specific epitope used for the solid phase) (conjugate 2) The assay procedure includes the following reaction steps : 1. Conjugate 1 (biotinylated polyclonal antibody to p24 HIV-1 Ag) is added into the microplate wells. 2. Serum samples to be assayed and controls are pipetted into the wells.
- If present, HIV antigens bind with the monoclonal antibody bound to the solid phase and the conjugate 1
- HIV-1 and/or HIV-2 antibodies, if any, bind to the antigens immobilised on the solid phase.
- Deposition of conjugate 1 and sample is validated through a colour change, from yellow-green to blue. 3. After incubation at 37°C then washing, conjugate 2 is added :
- Streptavidin react with biotinylated Ab-Ag-Ab complexes
- Peroxidase labelled, purified HIV-1 and HIV-2 antigens bind in turn to the IgG, IgM or IgA antibodies captured on the solid phase. 6 4. After incubation at 18-30°C the unbound conjugate 2 fraction is removed by washing. After incubation in presence of the substrate at room temperature (18-30°C) the presence of the complexed conjugate is shown by a change of colour. 5. The reaction is stopped and absorbances are read using a spectrophotometer at 450/620-700 nm. The absorbance measured on a sample determines the presence or absence of HIV Ag or HIV-1 and/or HIV-2 antibodies

2.2.5 HBsAg ELISA test

This was carried out using Diagnostic automation/ Cortez Diagnostics, INC kit by ELISA method.

2.2.6 HBsAg ELISA assay kit principle

The HBsAg ELISA Test kit employs an antibody sandwich ELISA technique where monoclonal antibodies unique to HBsAg, are pre-coated on polystyrene microwell strips. The serum or plasma sample is added together with a second antibody, the HRP Conjugate, (horseradish peroxidase) and directed against a different epitope of HBsAg. Throughout the time of incubation, specific immunocomplex that may have formed (indicating presence of HBsAg) is captured on the solid phase. After washing, to eliminate serum proteins and unbound HRP-conjugate, chromogen solutions containing tetramethyl-benzidine (TMB) and urea peroxide are added to the wells. Next, the colorless chromogens are hydrolyzed by the bound HRP-conjugate to a blue-colored product while in the presence of the antibody-antigen-antibody (HRP) sandwich immunocomplex. Halting the reaction with sulfuric acid, the blue color then turns yellow. The color intensity can be gauged proportionally to the amount of antigen captured in the wells, and to the amount in the sample, respectively. The wells remain colorless if the HBsAg result is negative.

2.3 Identification and Determination of Plasmodium Parasite Density

2.3.1 Staining of thick and thin smears

Two blood slides were prepared for each sample that came to the laboratory. Each slide had a

measured volume of 6 µl of blood for thick film and 2 µl for the thin film. Ten percent (1:9 ml) for 10 min and 3 % (3:97 ml) for 45–60 min fresh, working Giemsa stains were prepared with already prepared stock of Giemsa-staining solution and working Giemsa buffer prepared from buffer tablets. Thin and thick blood smear were stained with Giemsa after fixing the thin smear with absolute methanol. The 10% Giemsa stain was used to stain one of the two slides for preliminary slide reading to release results for participant management and treatment. The slide stained with 3% was given out by the slide coordinator to two competent, independent malaria microscopists. A positive smear was included with each new batch of working Giemsa stain for quality control[11].

2.3.2 Examination of thick/thin smears

The entire smear was first screened at a low magnification (10 X × 40 X objective lens) to detect suitable fields with even distribution of WBC (10–20 WBC/field). Smears were then examined using X100 oil immersion. At least 100 high power fields were examined before a thick smear was declared negative. *Plasmodium falciparum* parasites were counted per 200 or 500 leukocytes, which were used to estimate the parasite density per microlitre of blood. Thin films were examined to confirm the species identification on the thick film. Blood slide was declared positive when a concordant result was produced by two competent microscopists [11].

2.3.3 Estimation of parasite density

Parasite densities were recorded as a ratio of parasites to WBC in thick films. *Plasmodium* parasites were counted against 200 WBC on the thick film. Five hundred WBC were counted where less than nine parasites were counted after counting against 200 WBC. Where microscopists did the parasite counts in the thin film (against 2,000 red blood cells) as a result of heavy parasitaemia (greater or equal to 100 parasites per thick smear high power field), parasites counted were recalculated with 200 WBC. Parasite densities (parasite/µl of whole blood) were then calculated as follows:

= (Number of parasites counted/WBC counted) × WBC count/ µL of participant.

Also, parasite densities for all participants were calculated using assumed WBC of 5.0 × 10(9)/L, 6.0 × 10(9)/L, 8.0 × 10(9)/L and 10.0 × 10(9)/L of blood; all set by WHO to be used conveniently in facilities which lack the tools to determine patients' absolute FBC values [11].

2.4 Identification of *Mycobacterium tuberculosis* in Sputum Using Fluorescence Microscopy (auramine-rhodamine Staining)

2.4.1 Principle

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter . The filters and the dichroic beamsplitter are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen [12] In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images [12]

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective). These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).

2.5 Ethical Consideration

The proposal of this work was reviewed and approved by ethical and research committee of Baptist Medical center Saki-Nigeria before the commencement of this work. Informed consent was also obtained from each of the patient and control subjects.

2.6 Method of Statistical Analysis

The results obtained were subjected to statistical analysis using SPSS 18.0 to determine mean,

standard deviation, probability, student "t" test and level of significant at 0.05.

3. RESULTS

There was a significantly Higher plasma value of IL-10 in plasmodium infected patients with parasite density of 500-999 and ≥1000 than the control subjects with $p<0.05$. (Table 1, table 2, Fig 1 and Fig 2).

There was also a significantly lower mean plasma value of IL-10 in plasmodium infected patients with parasite density of 50-499 than those patients with parasite density 500-999 and ≥1000 with $p<0.05$ (Table 1, table 2, Fig 1 and Fig 2). However there was no significant difference in the plasma value of IL-2 in plasmodium infected patients based on parasite density with $p>0.05$ (Table 1, Table 2, Fig 1 and Fig 2).

Furthermore, there was no significant difference in the plasma value of IL-10 in plasmodium infected patients with parasite density of 50-499 compared with the control and those with parasite density of 500-999 compared with patients with plasmodium parasite density of ≥1000 with $p>0.05$) (Table 1, Table 2, Fig 1 and Fig 2). A frequency of : 12.0%(14) Anti-HCV seropositive, 6.9%(8) Anti-HIV seropositive, 19.8%(23) HBsAg seropositive, 13.8% (16) AFB positive patients and 4.3%(5) indeterminate results was obtained from the 116 Plasmodium infected patients initially recruited (Table 3).

4. DISCUSSION

This work has been used to reveal a significant alteration in the plasma value of IL-10 in plasmodium infection as discussed below.

There was a significantly higher plasma value of IL-10 in plasmodium infected patients with parasite density of 500-999 and ≥1000 than the control subjects. This finding agrees with the findings of Zeyrek et al.,(2006) [13] This could be associated with the physiological function of IL-10 as IL-10 is an anti-inflammatory cytokine that improves healing. Higher value of IL-10 in the patient could be as a result of inflammatory response aimed at providing immunity involving production of antibody specific to the invading organism to neutralize the effect of the invasion of plasmodium in the body system [14-18]. IL-10 has a multiple, pleiotropic, effects in immunoregulation and inflammation. IL-10 downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. IL-10 also enhances B cell survival, proliferation, and antibody production. It can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway [14-18].

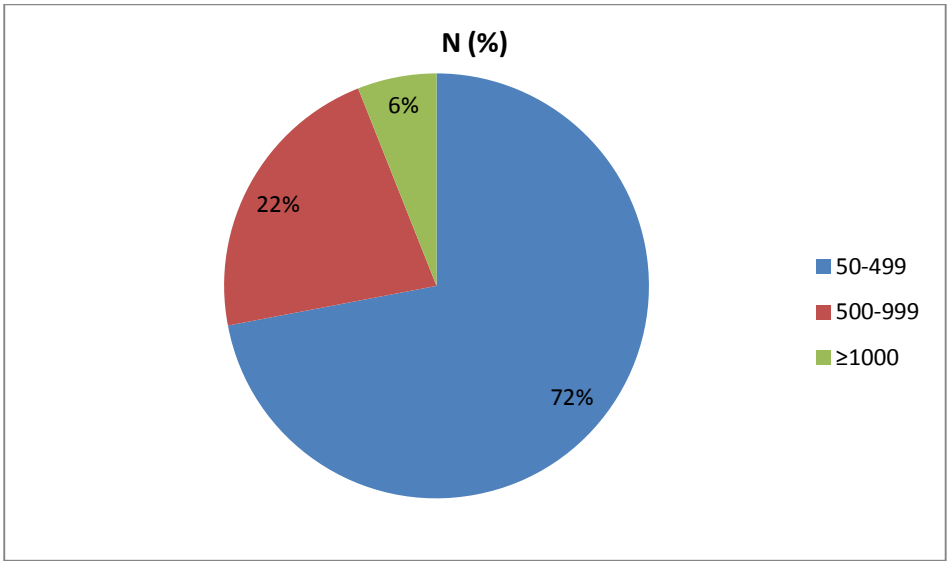

Fig. 1. Pattern of Plasmodium parasite density obtained in the subjects

Table 1. Plasmodium parasite density and immunochemical status obtained in the subjects

Parasite density			Immunochemical status					
Range/ µL	**N**	**Mean and standard deviation/ µL**	**Anti-HCV**	**Anti-HIV**	**HBsAg**	**AFB**	**IL-2 pg/ml**	**IL-10 pg/ml**
50-499	36 (72%)	292±10.0	Negative	Negative	Negative	Negative	3.4 ± 0.4	6.0±0.2
500-999	11(22%)	881±23.0	Negative	Negative	Negative	Negative	3.4 ± 0.3	7.8±0.3
≥1000	3(6%)	1300±25.0	Negative	Negative	Negative	Negative	3.6 ± 0.2	8.1±0.3
Control	50	--	-	-	-		3.3 ± 0.5	5.3 ±0.4

Table 2. Comparative analysis of plasmodium parasite density and immunochemical status obtained in the subjects

		Control vs 50-499	**Contro Vs 500-999**	**Control Vs ≥ 1000**	**50-499 Vs 500-999**	**50-499 Vs ≥ 1000**	**500-999 Vs ≥ 1000**
IL-2 pg/ml	"t"	-0.17	-0.17	-0.55	0.00	-0.44	-0.55
	"p"	0.45	0.43	0.05	0.5	0.35	0.32
IL-10 pg/ml	"t"	-1.56	-5	-5.6	-4.99	-5.82	-0.71
	"p"	0.13	0.02*	0.02*	0.02*	0.01*	0.27

**Significant with p<0.05*

Table 3. Frequency of Anti-HCV, Anti-HIV, HBsAg and AFB positive among the 116 plasmodium infected patients initially recruited

	Total number of Plasmodium infected patients initially recruited	**Anti-HCV seropositive plasmodium infected patients**	**Anti-HIV seropositive plasmodium infected patients**	**HBsAg seropositive plasmodium infected patients**	**AFB positive plasmodium infected patients**	**Indeterminate result**
N	116	14	8	23	16	5
Frequency in %	100%	12.0%	6.9%	19.8%	13.8%	4.3%

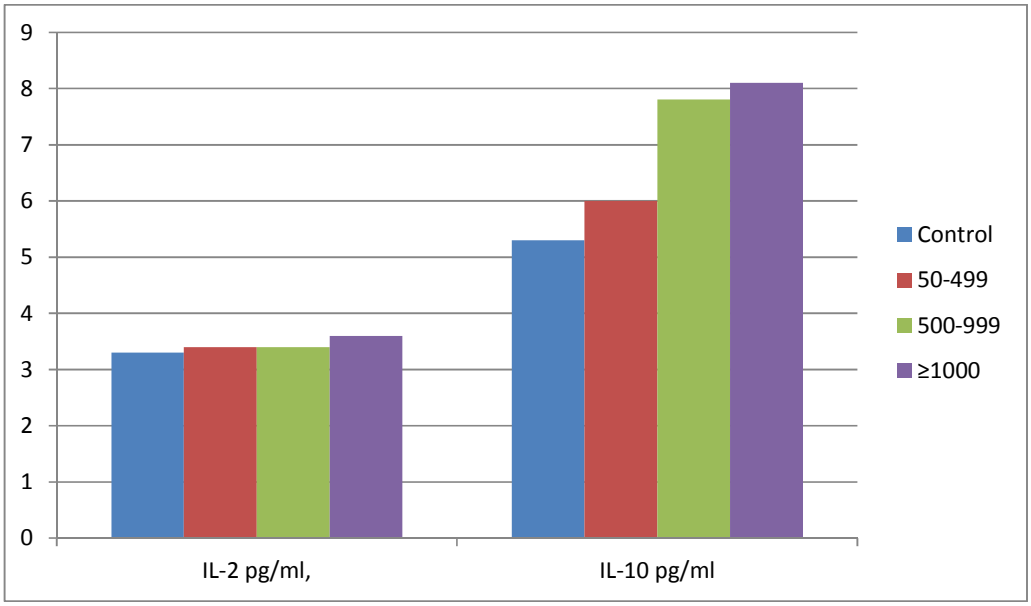

Fig. 2. Comparative description of Plasmodium parasite density, IL-2 and IL-10 obtained in the subjects

There was also a significantly lower mean plasma value of IL-10 in plasmodium infected patients with parasite density of 50-499 than those patients with parasite density 500-999 and ≥1000. This result is consistence with the findings of Zeyrek et al. (2006) [13] who reported that compared to controls, patients with a parasite count greater than 5000/microL had a significantly higher IL-1beta and IL-10 levels ($P < 0.05$). This findings could also be associated with the review of Mamoru et al. [19] that IL-10 is necessary for suppression of hepatic pathology or ECM in the host although IL-10 entails a risk of downregulation of protective immunity against malaria parasites. $CD4^+$ T cells of different kinds, such as pathological $CD4^+$ T cells, IL-10-producing $CD4^+$ T cells, or Treg cells, are induced during different kinds of Plasmodium spp infection [14-19]. A frequency of 12.0%(14) Anti-HCV seropositive, 6.9%(8) Anti-HIV seropositive, 19.8%(23) HBsAg seropositive, 13.8% (16) AFB positive patients and 4.3%(5) indeterminate results was obtained from the 116 Plasmodium infected patients initially recruited. Evidence of HIV, HCV, HBV and *M. tuberculosis* co-infection with *Plasmodium spp* has also been reported by previous authors [20-22].

5. CONCLUSION

Plasmodium parasitemia and increase in parasite density has been found to significantly increase the plasma value of IL-10 with no significant change in the plasma value of IL-2. There was also an evidence of HIV, HCV, HBV and *M. tuberculosis* co-infection with *Plasmodium spp* .

6. RECOMMENDATION

Routine evaluation of IL-10 and determination of possible coinfection in plasmodium infected patients is recommended for effective management.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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