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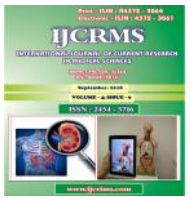

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Assessment of coagulation parameters in malaria infected pregnant women in Imo state Nigeria.

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Abstract

A cross-sectional prospective study was carried out on malaria infected pregnant women attending FMC Owerri , Imo state, Nigeria with the aim of assessing coagulation parameters, A total of 300 subjects within the age range of 18-45 years:100 infected pregnant women , 100 non- infected pregnant women and 100 non-infected non pregnant women (were recruited) for this study. Blood samples were collected from the subjects using standard method. These were analysed for coagulations parameters using semi- automated coagulation machine and ELISA respectively. Also malaria parasite infection was screened using rapid test kit (Bio) and confirmed microscopically. In addition, questionnaires were administered to the subjects to elicit demographic information about the consequences of malaria infection. The ages of the participants were analysed using percentages. All statistical analysis was performed using statistical package SAS version 9.4.The results were expressed as mean ± standard error of mean. Two tailed ANOVA and student t-test were used for comparison of differences in various groups and the level of significant was set at $P<0.05$.Pearson correlation was used for test of association of the various groups. The various results were represented graphically using overlay plot, box plot and correlation matrix to show nature of association. The data showed the mean age of the participant 29± 5.2 (40%), followed by 31-35 age range (26%). Most of the participants (78%) were in the third trimester at the time of study, while 14 and 8 were in the second and first trimester respectively. About three quarter of the participants (46%) were self employed, (16.7%) were civil servants and establishment, while the rest were workers in private, traders, students and unemployed. *Plasmodium falciparum* was the only specie identified. The partial prothrombin time with kaolin (PTTK) was significantly increased compared with the malaria pregnant women and non malaria pregnant women ($P<0.05$).. Protein C and Protein S were remarkably decreased in infected malaria subjects compared to non-infected subjects. Protein C and S are the major anticoagulants and inflammatory markers in malaria infection, thereby ameliorating thrombosis in pregnancy.

Keywords: coagulation, pregnant women, ELISA, *Plasmodium falciparum*, PTTK .

Introduction

Malaria, a condition caused by infestation with Plasmodium parasite specie, is a major public health problem globally especially in developing countries causing considerable morbidity and mortality especially in sub Saharan Africa where it accounts for up to 1 million death per annum (Murray *et al*., 2012).

Pregnant women are at high risk of being infected with malaria owning to the ability of the parasite to adhere to trophoblastic villous epithelium and sequester in the placenta which could eventually lead to poor pregnancy outcome. It is estimated that over 200,000 infants die annually in sub-Saharan Africa as a result of their mother becoming infected with malaria during pregnancy (Steketee *et al*., 2001). Malaria during pregnancy can lead to maternal and foetal adverse effects, mainly anaemia, cerebral malaria, hemorrhage and low birth weight.

Haemostasis is the regulation of blood loss in the case of injury to the vain and artery and the dissolution of excessive blood clot in cases of thromboembolism. Previous studies indicate that Plasmodium falciparum malaria especially the severe form can lead to an impairment of the coagulation system which correlates with pro-inflammatory cytokines. Fibrin deposition is an important feature of placental malaria infections. Furthermore, it has been shown that excessive fibrin deposition in the infected placenta occurs in association with dramatic upregulation of tissue factor, the initiator of the extrinsic pathway of coagulation on infiltrating monocytes. Malaria has been associated with changes in coagulation and fibrinolytic factors of blood.

Thrombocytopenia is a common finding in patients with Plasmodium falciparum malaria. Some studies with human indicate that there is undoubtedly increased coagulation activity in malaria. Significant elevations in plasma levels of thrombin, antithrombin complexes, fibrin degradation products and D-dimers have been reported (Holst *et al*., 1999; Moxon, 2015). Although mild prolongation in both the prothrombin time and activated thromboplastin have been noted; plasma fibrinogen level typically remain with normal range (Amged *et al*., 2015). Finally in keeping with this state of coagulation activity, significant reductions in plasma anticoagulant factor (including antithrombin, protein-C and protein-S) have also been observed in patient with malaria.

Pregnancy is associated with changes in haemostasis, including an increase in the majority of clotting factor, a decrease in the quantity of natural anticoagulants, and a reduction in fibrinolytic activity (Bremme, 2003).The platelet count decreases in normal pregnancy, possibly due to increased destruction and hemodilution, with a maximal decrease in the third trimester (O'Riordan and Higgins, 2003). As most coagulation factors increase in normal pregnancy, the prothrombin time (PT) and the activated partial thromboplastin time (APTT) may be shortened. Laboratory-based screening is used routinely to assess coagulation status in obstetric patients. The tests consist of platelet count; PT, APTT, D-Dimer, and plasma fibrinogen levels.

Aim

The study is aimed at assessing coagulation and fibrinolytic parameters in malaria infected pregnant women in Imo State, Nigeria.

Specific objectives

1. To compare the difference between haemostatic parameters (prothrombin time, activated partial thromboplastin time, fibrinogen, protein-C and protein-S), in pregnant women infected with malaria, non-infected and non pregnant women in Imo State of Nigeria.
2. To compare the difference between the haemostatic parameters (prothrombin time, activated partial thromboplastin time, fibrinogen, protein-C and protein-S), in malaria infected pregnant women, non infected pregnant women and non infected non pregnant women in Imo State of Nigeria.

Materials and Methods

Study area

This study was carried out in Federal Medical Centre Owerri in Imo State, Nigeria.

Study population and sample size

A total of 300 subjects between the age of 18-45 years were recruited for the study. 200 pregnant women attending maternity clinic at Federal medical Centre Owerri and 100 non pregnant women were eligible for the study.

The sample size was obtained using the formula by Naing *et al.*, 2006. Prevalence rate of malaria infected pregnant women is 74.6% (Ohalete *et al.*, 2011).

$$n = z^2 \times P(1-P)/d^2$$

Where
n = Sample size
p = prevalence rate 74.6%
z = confidence interval 95% - 1.96
d = Degree of accuracy- 0.05
$N = 1.96^2 \times 0.746(1-0.746)/0.05^2$
= 288

Experimental design

A cross sectional prospective study was carried out on 3 groups.

Group 1 =100 Malaria Infected Pregnant Subjects,
Group 2 =100 Non Malaria Infected Pregnant Subjects,
Group 3 =100 Non Malaria non Pregnant Subjects.
An oral consent was gotten from the patients after which a structured questionnaire was administered to all respondents who was also part of clinical study.

Ethical consideration

A letter of introduction was secured from the Head of Department, Medical Laboratory Science of River State University. This letter was submitted to the ethical committee of Federal Medical Centre Owerri to seek for ethical approval to carry out the study. After all considerations the Ethical committee approved my request.

Sample collection

About 5.5ml of venous blood was drawn from each participant using standard veno puncture techniques. 2.0mL dispensed into 0.25mL of 3.2% trisodium citrate anticoagulated container for coagulation studies and 3.5mls dispensed into a plain container to obtain serum. The sample in the citrate anticoagulated test tube was centrifuged for 5 minutes at 3000 rpm to separate the plasma. The collected samples were analysed immediately for coagulation test.

Laboratory procedures

All reagents were commercially purchased and the manufacturer's Standard Operating Procedures (SOP) were strictly followed.

A) Malaria Estimation Using Rapid Test kit

As modified by SD BIO LINE One Step Malaria antigen P.F (HRP-II) rapid kit was used.

Test Procedure

The kit was allowed to equilibrate at room temperature. The test device was opened for and labeled for each patient. The specimen was collected with the aid of capillary pipette provided and then transferred into the round specimen well. Four drops of assay diluents was dispensed into the diluents well. The kit was left on a flat bench for a period of 15 minutes before taking result.

B) Malaria Parasite Identification using Giemsa Staining Technique (cheesbrough, 2010).

Methodology

A drop of blood was placed on the slide to cover the diameter 15-20mm. The blood was smeared evenly on the slide to obtain a thick film and then allowed to air dry with the slide in a horizontal

position. Before staining, the stock giemsa stain was diluted in 1:10 dilution using phosphate buffer at pH 7.2 .The working solution of the giemsa stain was used to cover the dried thick film for 30 minutes and at the end of the staining period, water was used to gently flush the stain off the slide. The slide was rinsed briefly in gently running tap water and the under surface of the slide blotted dry to remove excess stain. It was left to air dry in a vertical position and then viewed microscopically using x40 and x100 objectives.

C) Hematological parameter estimation

The EDTA blood was measured on a fully automated haematological analyser, a five part auto analyser able to test 19 parameters per sample using the Sysmex® KX-21N autohaematological analyser. Standardization, calibration of the instrument and processing of the sample was done according to the manufactures instruction.

Procedure

An EDTA anticoagulated blood was well mixed, inserted into the probe. The button was pressed and 0.02ml of blood was aspirated. After a period of 1 minute the heamatological results were displayed in the screen and printed with the aid of the printer.

D) Prothrombin Time Estimation (Cheesbrough, 2010)

As modified by GIESSE Diagnostics.

Procedure

All reagents were prewarmed at 37^{oC}. 100uL of tri sodium citrate anticoagulated blood was dispensed in a plastic tube. It was incubated for 2 minute at 37^{O}C. 200uL of reagent was added. Time taken to clot was read and recorded.

E) Activated Partial Thromboplastin Time Estimation (Cheesbrough, 2010)

As modified by GIESSE Diagnostics was used.

Procedure

All reagents were prewarmed at 37^{o}C. 100uL of tri sodium citrate anticoagulated blood was dispensed in a plastic tube. 100uL of reagent was added and mixed together. It was incubated for 4 minute at 37^{o}C. 100uL of calcium chloride was added. Time taken to clot was read and recorded.

F) Fibrinogen assay (Clauss, 1957)

As modified by GIESSE Diagnostics was used.

Procedure

Samples and controls were dilluted 1:10 with imidazole buffer (50uL + 450uL). 200uL of predilluted samples were pipetted into a plastic tube and incubated for a period of 5 minutes at 37^{oC}. 100 uL of Bovine thrombin was added and the time taken to clot was recorded.

G) Protein C assay

Commercial Kit by MELSIN diagnostics was used. Catlogue Number: EKHU-1392.

Procedure

50 uL of standards were pipetted into the standard wells. 10uL of test serum were pipetted into each sample well. 40uL of sample dilluent was added to the sample well. 100 uL of HRP-conjugate reagent was added to all wells, covered with an adhesive strip and incubated for 60minutes at 37oC. It was washed for four times. 50uL of chromogen solution A and 50uL of chromogen solution B was added to each well. They were mixed incubated for 15 minutes at 37oC. 50uL of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450nm wavelength within 15 minute.

Calculation

A standard curve of optical density against concentration of standard was plotted and the concentration of the tests determined from there.

H) Protein S assay

Commercial Kit by MELSIN diagnostics was used. Catlogue Number: EKHU-1232.

Procedure

50 uL of standards were pipette into the standard wells. 10uL of test serum were pipette into the each sample well. 40uL of sample dilluent was added to the sample well. 100 uL of HRP-conjugate reagent was added to all wells, covered with an adhesive strip and incubated for 60minutes at 37^{o}C. It was washed for four times. 50uL of chromogen solution A and 50uL of chromogen solution B was added to each well. They were mixed incubated for 15 minutes at 37^{o}C. 50uL of stop solution was added to each well. Optical density of the samples was read in a microtiter plate reader at 450nm wavelength within 15 minute.

Calculation

A standard curve of optical density against concentration of standards was plotted and the concentration of the tests determined from there.

Statistical analysis

All statistical analysis was performed using Statistical Package SAS VERSION 9.4. The results were expressed as mean plus or minus standard error of mean in tabular form. Analysis of variance (ANOVA) and student t- test were used for comparism of differences in various groups.

All test performed were two tailed and the level of significant was set at $p<0.05$.

Results

The result was designed to evaluate the assessment of malaria in pregnancy on coagulation markers. They were displayed in tables, graphical plots and matrix accordingly as shown below:

Table 1: Demographic Characteristics of Study Subjects and Mosquito Control Methods Used

Characteristics	n	%	95% Confidence Interval
Age Group (years)			
18 – 24	30	20.0	14.4-27.1
25 – 30	60	40.0	32.5-48.0
31 – 35	39	26.0	19.6-33.6
36^{+}	21	14.0	9.3-20.5
Mean ± SD (years)	150	29.5±5.2	28.7-30.4
Trimester			
1st	8	8.0	4.1-15.0
2nd	14	14.0	8.5-22.1
3rd	78	78.0	69.9-84.9
Parity			
Prime	9	9.0	4.8-16.2
Second	35	35.0	26.4-44.7
Multi	56	56.0	46.2-65.3
Occupation			
Civil Servant	25	16.7	11.6-23.4
Worker in Private	10	6.7	3.7-11.8
Establishment	25	16.7	11.6-23.4
Trader	5	3.3	1.4-7.6
Self-employed	69	46.0	38.2-54.0
Student	13	8.7	5.1-14.3
Unemployed	3	2.0	0.0-5.7
Other			

Percentages may not add up to a 100 due to rounding

Table 1 shows the demographic characteristics of study subjects and control method used. The mean age of the participants was 29.5±5.2. Majority of the pregnant women were in the range 25-30 years of which accounted to 40% followed by 31-35 years (26%). The pregnant women were grouped according to trimester. Majority (78%) of the participant were in their third trimester and the least (8%) in their first trimester. With respect to parity, 9% of the pregnant women were primegravidae followed by secondigravidae (35%) and the highest was the multigravidae (56%).

46% of the pregnant women were self employed, 16.7% civil servant, 8.7% students, 6.7% workers in private, 3.3% traders and 2.0% unemployed. Out of 150 participants, 51.3% used bed nets, 31.3% window net and 17.3% mosquito repellants.

Table 2: Comparisons of Mean ± SEM of Coagulation Parameters by Treatment

	Treatment			Test Statistics
Parameter	**MP^{+}**(n=100)	**MP $^{-}$**(n=100)	**Control**(n=100)	**P-Value**
Prothrombin Time	13.11±0.20	13.23±0.26	13.59±0.22	0.303^{ns}
PTTK	28.27±0.57 ^{a}	28.30±0.71^{a}	25.73±0.36^{b}	0.002*
INR	1.09±0.02	1.10±0.02	1.16±0.02	0.061^{ns}
Protein C	1.02±0.11 ^{a}	1.12±0.12 ^{a}	2.13±0.39^{b}	0.002*
Fibrinogen	274.46±6.71	273.84±7.81	270.83±3.81	0.910^{ns}
Protein S	8.93±0.44 ^{a}	9.60±0.65 ^{a}	15.80±2.39^{b}	0.002*

Within parameter, mean ± SEM with different superscript are significantly different at p<0.05.

a, b, c - Indicates significant differences

Significant level - *P < 0.05, **P < 0.001, ***P<0.0001

ns - Not significant (P > 0.05)

Table 2 shows the comparisons of mean and standard error of mean (SEM) of Coagulation and fibrinolytic parameters according to the three groups.

Prothrombin time was lower in the infected and uninfected women (13.11 ±0.29sec & 13.23 ±0.26 sec respectively) than the control (13.59 ±0.22sec) and the differences among the means were non significant (P=0.303). Partial thromboplastin time test was higher in pregnant women with 28.27 ±0.57 sec in infected women and 28.30 ±0.71 sec in uninfected women when compared with the control (25.73 ±0.36sec). The differences among their means were significant. Protein C was decreased in infected and uninfected pregnant women (1.02 ±0.11 and 1.12 ±0.12 respectively) than the control (2.13 ±0.39) and there was a statistical significantly difference among their means. Fibrinogen concentration was increased in the pregnant women with 274.46 ±6.71mg/dL in infected women and 273.84 ±7.81mg/dL in uninfected women when compared with control (270.83 ±3.81mg/dL) but difference among their means was not significant. Protein S concentration was decreased in malaria infected and uninfected women (8.93 ±0.44 and 9.60 ±0.65 respectively) than the control (15.80 ±2.39) with a difference among their means.

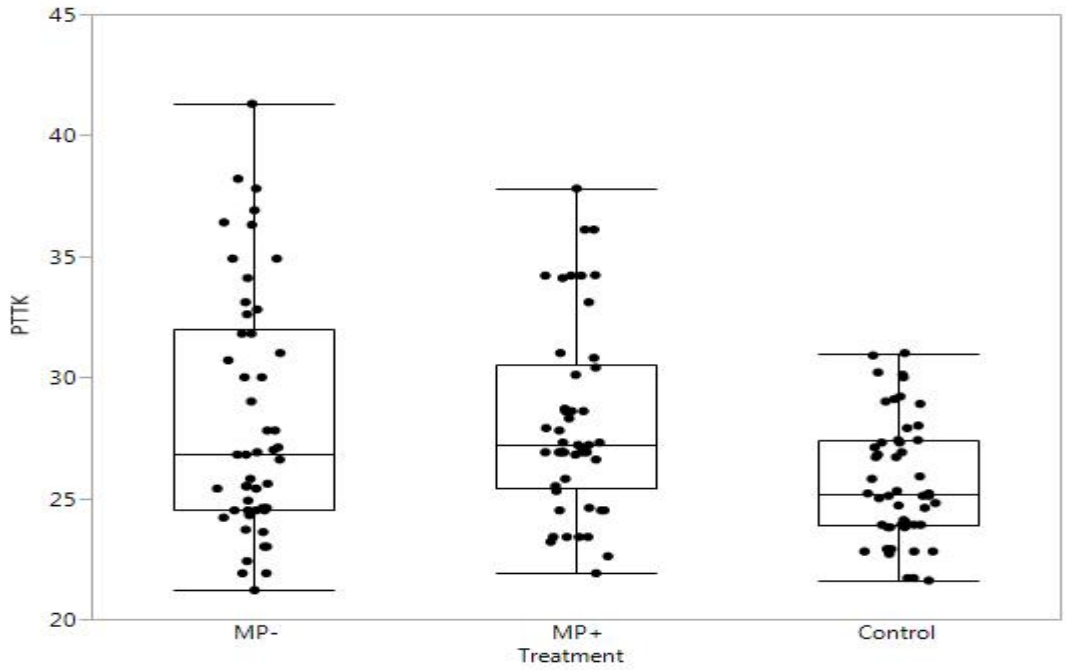

Figure 1: PTTK by Treatment

Discussion

The age-related prevalence of malaria infected pregnant women showed a decrease in infection with increase in age from 60% in women in the age group 25-30 years to 21% in those in the 35-above years.

It was observed that women in their first trimester (8%) had lesser prevalence than those in their second (14%), and third (78%) trimesters respectively.

In relation to parity, the prevalence of parasitaemia, was higher among the multigravidae (56%) than the primigravidae (9%) and Secondigravidae (35%). This is because while those findings are of the view that parasitaemia was significantly higher in primigravidae than in multigravidae , indicating a strong relationship between parity and malaria infection with mean parasite density levels decreasing as the number of gestation increased thus confirming that the African primigravidae remain unquestionably the most susceptible (WHO 2003) but this is contrary to this particular work which showed that the multigravidae are the most susceptible group This is further explained by (WHO, 2002) that in the first and second pregnancies, women are especially vulnerable to *P. falciparum* parasitaemia.

Pregnancy is associated with changes in haemostasis, including an increase in the majority of clotting factors, a decrease in the quantity of natural anticoagulants and a reduction in fibrinolytic activity (O'Riordan & Higgins, 2003).

Possible causes of reduction in platelet count include reduced platelet survival from peripheral destruction (by immune, consumptive, or other mechanisms), enhanced splenic uptake or sequestration, and decreased platelet production. Recently, it was shown that thrombocytopenia in early malaria is associated with vWF-mediated GP1b shedding, a process that may prevent excessive adhesion of platelets and parasitized erythrocytes (de Mast *et al*., 2010). This can also be due to platelet activation, splenic pooling and a decreased platelet life span.

Prothrombin time is shortened in pregnant women compared with the control group. This could be as a result of decrease in FII, FV, FVII, FX and fibrinogen. Some nutritional deficiencies and/or liver disease will decrease these factors prolonging the prothrombin time. In our study activated partial thromboplastin time are prolonged in pregnant women than the control. This may be as a result of the presence of an antiphospholipid antibody (APLA), such as lupus anticoagulant. Patients with APLA are prothrombotic. This can also be due to deficiencies of FVIII, FIX, FXI and FXII.

From our study there is decrease in PT and APTT in pregnant women infected with malaria when compared with those without malaria and control. This is because malaria stimulates coagulation system. The stimulation of the coagulation system is caused by various procoagulants present during malarial infection. The sources of the procoagulants are exposed phosphatidylserine on the cell surface of infected erythrocytes, the lysis of activated platelets together with their secretory products, and the tissue factor (TF) released from damaged vascular endothelial cells. Furthermore, certain substances that are released during severe malarial infection - namely tumor necrosis factor α (TNF α) and histamine - are additional factors that promote fibrin formation. The intrinsic pathway of the coagulation has also been shown to be activated in severe malaria (Clemens *et al*., 1994). In turn, this may cause activation of the complement system and release of bradykinin and PMN-derived elastase that could contribute to the pathogenesis of severe malaria.
There is hyperfibrinogenaemia in pregnant women. Fibrinogen also increases during pregnancy with levels at term 200% above pre-pregnant levels (Bremme, 2003).

There is a reduction in both protein C and S in pregnant women when compared with the control. This is due to decreases in t-PA activity, which remains low until 1-h postpartum when activity returns to normal. This reduction is due to the gradual, eventually threefold, increase in plasminogen activator inhibitor-1 (PAI-1) and the increasing levels of plasminogen activator inhibitor-2 (PAI-2) (O'Riordan & Higgins, 2003). The placenta produces PAI-1 and is the primary source of PAI-2. PAI-2 levels at term are 25 times that of normal plasma (Kruithof *et al*., 1987).

This was also in line with the work of Mohanty *et al*., (1997). The reduction in the levels of protein C and protein S is attributed to increased consumption due to microvascular thrombosis rather than to reduced synthesis in the liver. Thus, activation of protein C may be a control mechanism related to host defense in malaria as it is in sepsis. This can also be as a result of increase in Plasma levels of plasminogen activator inhibitor-1 (PAI-1)

Conclusion

Procoagulants (Protein C and S) are important anticoagulants and anti inflammatory pathway triggered in response to the generation of excess thrombin production therby emiliorating thrombosis in pregnancy. Protrin C and S are the majornatural anticoagulant

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