Alterations in some coagulation indices among adults infected with *Plasmodium Falciparum* attending a Tertiary Health Facility in Nnewi, Anambra State, Nigeria.

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Abstract

Background: Malaria infection still remains a heavy burden in the tropics and has been the cause of childhood and maternal mortality and morbidity. Malaria infection is said to alter coagulation parameters bringing about a derangement in the coagulation mechanism.

Objective: The aim of this study was to determine the effect of *p. falciparum* on Platelet counts, Prothrombin Time, and Activated Thromboplastin Time in infected adults attending a Tertiary Health Facility in Nnewi, Anambra State, Nigeria. **Methods**: This longitudinal study enrolled 270 subjects that met the defining criteria, they were then grouped into 3 based on malaria parasite preset cut off of ≥ 1000 parasites x 10^9/l, thus, group one (100) had count above the cut off while group two(100) had counts below. Group three (70) tested negative to *plasmodium* antigen therefore served as control. Post treatment sample was also collected from test group. Questionnaires were administered and 6mls of blood drawn from each subject, 1.5mls was dispensed into EDTA for platelet count and thick film for parasite quantification, while 4.5mls was dispensed into 0.5ml of 32g/l trisodium citrate for coagulation tests using platelet poor plasma. **Results**: There was a progressive drop in platelet counts with increase in parasite density (P < 0.05). Group one had platelet count of 140.0 ±28.77 x 10^9/l, as against 201.42 ±41.08 x 10^9/l in group two and control (264.36 ± 49.20 x 10^9/l)), this trend was also observed seen with PT and APTT (seconds) were progressive significant prolongation in time was observed as the parasite count increases (P<0.05). Thus PT and APTT (seconds) for group one were 25.09±6.24; 43.20±8.39, group two were 22.26 ± 2.84; 34.88± 8.25, and control 20.09 ± 2.87, 27.94± 5.27. The values of the tested parameters reverted to normal value post treatment (p<0.05).

Conclusion: Coagulation mechanism is altered in malaria and the degree of alteration is proportional to the parasite density, this could precipitate bleeding in infected individuals.

Keywords: Malaria Parasite, Prothrombin Time, Activated Thromboplastin Time, Platelet count

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Introduction

Malaria is a global problem (1) with sever health hazard placing aheavy toll on lives and economy (2). It accounts for at least 1 million deaths every year and more than 60% of hospital visitation in endemic regions(1,3). It is prevalent in more than 90 countries (3) especially in Sub-Saharan Africa (1). Of all the species affecting man, *P falciparum* infection records the highest morbidity and mortality because of its varied and dreaded complications, often resulting in multiple systems or organs involvement, notably renal, hepatic, cerebral, pulmonary, metabolic and haematologic(1, 2) Increase in coagulation activity in malaria appears to be a feature in malaria[2] this may be triggered by activation of coagulation cascade as a consequence of inflammation which is an essential part of the host defense(4). This study focuses on the changes affecting thrombocytes and the coagulation proteins determined by PT and APTT as previously recommended (5).The major changes of thrombocytes during malarial infection are reduction in counts and dysfunction.(6-8) Thrombocytopenia had been reported to occur due to destruction of peripheral platelet as a result of secreted IgM and IgG in inflammatory response(6-8) and consequent attachment to the platelets(8), activation of complement (9), and splenic sequestration(10), or due to consumption of platelets as a part of disseminated intravascular coagulation (10,11). Platelet dysfunction is believed to be occasioned by increase in platelet activity in the first instance and subsequent drop inactivity consequent to consumption (12-13).Some other researchers reportsthe release of procoagulants from parasitized RBC membrane (13) as well as intravascular lyses of RBC and platelets (15) as mechanism involved. So the coagulation system inrecent times is recognized to play a vital role in malaria pathology (11), and this is evidenced by reports on several studies on altered levels of coagulation parameters in infected individuals (15,16).The report of Smith *et al* that coagulation parameters revert back to normal supports this belief (17). Yet determinates of coagulation had been lacking in laboratory investigations ofthose infected by *P. falciparum.* This study was therefore designed to determine the effect of *p. falciparum* on Platelet counts, Prothrombin Time, and Activated Thromboplastin Time in infected adults living in Nnewi, Anambra State.

Materials and Methods

Study site

This study was carried out in a Tertiary Hospital in Nnewi, Anambra State, South- East Nigeria. Nnewi is located at 6.02^0 North latitude, 6.915^0 East longitude and 149 meters elevation above the sea level with a population of 769,500 (according to 2006 National Population census). It falls within the tropical rain forest,the rainy season stretches from March to October and dry season from November to February. Nnewi has bi-peak malaria transmission in March and November corresponding the onset and cessation of rain(18). The temperature range is between 20^0C and 36.5^0C. The climatic weather, poor sanitary conditions and the rain forest vegetation, create a favourable breeding site for vectors.

Study population/Study Protocol

This longitudinal study comprised of patients who presented at GOPD of the Hospital with history of fever, headache, and/ or with axillary temperature of $\geq 37.5^0C$in the last 24 hours and who hadn't taken any anti- malaria drug, and in whom test for malaria parasite had been requested by the attending Clinician. The subjects had drops of blood collected from them for Rapid Diagnostic Testing(RDT) and thick peripheral blood film(PBF). The thick PBF was stained with 10% Giemsa stain and result was considered negative if no malaria parasite was seen after examining at 100 high power fields.

Those that tested positive were enrolled into the study. A total of 200 subjects formed the test group. They were further grouped into 2 based on the

preset cut-off parasite density of 1000 parasites x 10^9/las prescribed by WHO 2000 (19). Thus Group one (100) had malaria parasite count $\geq$ 1000 parasites x 10^9/l, and Group two (100) had count < 1000 parasites x 10^9/l.Control were 70 apparently healthy individuals who were negative to *P. falciparum* antigenby PBF examination or RDT, selected from staff and students of the same hospital. Post treatment sample was also collected from the test groupfrom day 2-4post-treatment and ACTs was the anti-malarial administered. Almost half of subjects were lost during follow up.The subjects were within the ages of 18-65 years and demographic data collected through questionnaire.

Ethical consideration
This was sought and obtained from the institutional ethics committee and informed consent obtained.

Blood Sample Collection
Blood of 6mls volume was collected from each subject that met the defining criteria before and post treatmentand also the control and 4.5mls was dispensed into 0.5ml of 32g/l trisodium citrate and platelet poor plasma was obtained by centrifugation for PT and APPT assay: average of duplicate reading was taken. The remaining 1.5 ml was dispensed in EDTA bottle for platelet and parasite counts.

Methods:
Procedure for thick film for malaria parasite determination
A small drop of blood was dropped at the center of a clean slide and spread out with the corner of another slide to cover an area of about four times its original area. The film was allowed to dry for one hour. The slides were stained using 10% Giemsia stain an alcohol-based Romanowsky stain atpH 7.2 and stained for 10 minutes then washed off with clean buffered water at pH7.2. The back of each slide was wiped and placed on a draining rack to air dry and examined with 100x objectives with 7x eyepieces (20).

Estimation of Parasite Density Using Thick Film
A part of the thick film where white cells were evenly distributed and the parasites well stained with 10% Giemsia stain was selected, using the oil immersion objective, one hundred white blood cells was systematically counted, at the same time the number of parasite in each field was counted (20).

Calculations
The number of parasites per microliter of blood was calculated as follows:

Parasite $x10^9$/l of blood=

$$\frac{\text{WBC count x parasite count against 100 WBC}}{100}$$

Activated partial thromboplastin time (APTT) assay (from Agappe diagnostics Ltd (Kerala, India)

Principle
In the presence of calcium ions cephaloplastin activates coagulation factors of intrinsic pathway in plasma leading to clot formation. Clotting time is proportional to concentrations of factors VIII, IX, XI and XII as well as common pathway factors II, V and X. as the reagent is prepared using one single species rabbit brain, it has the required sensitivity to be used in heparin assays, also has the sensitivity for factors VIII and LA (21).

Assay Procedure
Reagent vials were swirled gently before use. Calcium chloride $CaCl_2$ (Reagent one) was Pre-warmed at 37^0C. 100µl of test plasma was pipetted into test cuvette at 37^0C. 100µl of pre-warmed Activated Partial Thromboplastin Time reagent (Reagent two) was pipetted into test cuvette. Both the test plasma and reagent two were mixed well and incubated at 37^0C for 3 minutes. 100µl pre-warmed $CaCl_2$ (Reagent one) was forcibly added into the test cuvette. The timer was simultaneously started and clotting time in seconds recorded.

Prothrombin Time (PT-INR) Assay (fromAgappe diagnostics Ltd (Kerala, India)
Principle
Tissue thromboplastin in the presence of Ca++ activates extrinsic pathway of human blood

coagulation cascade. Activation time is proportional to the concentration of individual clotting factors taking part in the coagulation cascade. This assists in estimating cause and extent of haemorrhagic disorder. When thromboplastin reagent is added to citrated plasma, clotting cascade is initiated forming gel clot. The time required for clot formation would be prolonged if there is deficiency of factor (s) activity in the extrinsic pathway of the coagulation cycle (21).

Assay Procedure

Reagent vials were swirled gently before use. Prothrombin reagent was Pre-warmed at 37^0C for ten minutes. 100µl of test plasma was pipetted into test cuvette and incubated at 37^0C for three minutes. Then 200µl of pre-warmed prothrombin reagent was forcibly added into the test cuvette. The timer was simultaneously started and clotting time in seconds recorded and International Normalized Ratio extrapolated. (INR) = R^{ISI**} where,

R (Prothrombin Ratio) = <u>Mean PT of the patient's plasma (sec)</u>

                         Mean Normal PT of the control (sec) *

> *Pooled Plasma from control group was used to obtain the mean normal PT.
> **ISI value of the reagent = 1.05

Statistical Analysis (Using SPSS 20)

Data analysis was done using statistical package for social science (SPSS) version 20. The results were expressed as mean ± S.D. Inferential statistics done using way analysis of variance (ANOVA) for comparison of multiplemeans and Turkey Post Hoc tests for intragroup comparison. P <0.05 were considered statistically significant.

Results

Table1. The mean platelet count was significantly lower in group one subjects $(140.0 \pm 28.77 \text{x} 10^9/\text{L})$ compared to group two subjects $(201.42 \pm 41.08 \text{ x} 10^9/\text{L})$ and control subjects $(264.36 \pm 49.20 \text{ x} 10^9/\text{L})$ (p<0.05), while the mean PT (INR) was significantly higher in group one subjects (1.45 ± 0.39) compared to group two subjects $(1.28\pm 0 .17)$ and control subjects (1.15 ± 0.17) (p<0.05). Similarly, PT (seconds) was significantly prolonged in group one subjects (25.09 ± 6.24) compared with group two subjects (22.26 ± 2.84) and control subjects (20.09 ± 2.87) (p<0.05).The mean APTT (sec) was significantly higher in group one subjects (43.20 ± 8.39) compared with group two subjects (34.88 ± 8.25) and control subjects (27.94 ± 5.27) (p<0.05).

Table 2: There was no difference between the Pre-treatment, Post- treatment and control values of tested parameters (p<0.05). Group One had: Platelet $(\text{x} 10^9/\text{L})$ =275.30 ± 43.53; INR =1.16±0.18, PT (sec)=20.35±2.96 and APTT (sec)=28.81±4.62 while Group Two: values were Platelet $252.09 \pm 52.86 \text{x} 10^9/\text{L}$; INR 1.12±0.17, PT 19.79±2.77(sec), APTT 26.97±5.82 (sec) and Control Platelet was $264.36 \pm 49.20 \text{ x} 10^9/\text{L}$, INR=1.15 ± 0.17, PT =20.09 ± 2.87and APTT=27.94 ± 5.27.

TABLE 1: Comparison of the Pretreatment Parameters for the various groups using ANOVA

Groups	Group one n=100	Group two n=100	Control n=70	F-value	P-value	Group one vs. two	Group one vs. control	Group two vs. control
Platelet $(x10^9/L)$ Mean±SD	140.0 ± 28.77	201.42 ± 41.08	264.36± 49.20	206.44	0.000**	0.000**	0.000*	0.000*
PT(INR) Mean±SD	1.45 ±0.39	1.28 ± 0 .17	1.15± 0.17	26.80	0.000**	0.000**	0.000*	0.007*
PT(secs) Mean±SD	25.09 ± 6.24	22.26 ± 2.84	20.09± 2.87	27.32	0.000**	0.000**	0.000*	0.005*
APTT(secs) Mean±SD	43.20 ± 8.39	34.88 ± 8.25	27.94± 5.27	84.19	0.000**	0.000**	0.000*	0.000*

*values differ significantly from control (p<0.05). **values differ significantly between Group one and group two subjects (p< 0.05)

TABLE 2: Comparison of the Pretreatment Parameters for the various groups using ANOVA

Parameters	Group one (n=37) Mean ±SD	Group two (n=33) Mean ± SD	Control (n=70) Mean ± SD	F-value	p-value
Age (years)	39.81 ± 10.38	36.39 ± 13.76	37.23 ± 12.70	1.181	0.242
Platelet(x 10^9/l)	275.30 ± 43.53	252.09 ± 52.86	264.36± 49.20	2.013	0.048
PT (INR)	1.16 ± 0.18	1.12 ± 0.17	1.15± 0.17	0.978	0.331
PT (secs)	20.35 ± 2.96	19.79 ± 2.77	20.09± 2.87	0.819	0.416
APTT (secs)	28.81 ± 4.62	26.97 ± 5.82	27.94± 5.27	1.472	0.146

Values differ significantly from control (p<0.05)

Discussion

In this longitudinal study aimed at determining the effect of *Plasmodium Falciparum* on some coagulation indices in infected adults in a tertiary health institution in Nnewi, we observed that platelet count was significantly lowered in malaria infected subjects compared to control; the higher the degree of parasitemia the lower the platelet counts. We therefore report that the platelet count has a direct relationship with the parasite count in our study population.This observation is consistent withseveral reports (22,23,24,25,26,27, 4). In the group with ≥1000 parasite counts (group one), thrombocytopenia was noted in addition. The reason for thelowering in platelet population as suggested from earlier work could be said to be multifactorial; while Kelton *et al* and Sorensen *et al* in their independent studies attributed it toimmune mediation resulting in a rise inmacrophage colony stimulating factor (M-CSF) and concomitant increase in activitythereby inducing platelet destruction(6,7). Srichaikul*et al*on the other hand believed it is due to sequestration in the spleen consequent to complement coating (9).Two other independentworkers in their reporttrace the cause to oxidative stress damage since superoxide-dismutase and glutathione peroxidase antioxidant enzymes activity were found to be raised in malaria patients when compared to those of healthy subjects (28, 23). de Mast *et al* in their study hypothesized that thrombocytopenia in malaria is associated with glycoprotein 1b (GP1b) shedding in the absence of systemic platelet activation and consumption coagulopathy (29).

This study reports in addition that the prothrombin time (PT) and activated partial thromboplastin time (APTT) was significantly prolonged in our test subjects when compared tocontrol group. We further observed that the PT and APTT time was more prolonged in subjects with higher parasite counts than in those with lower counts. This finding is consistent withseveral previous works and reports (30,25,4,31). The prolonged PT and APTT time had been suggested to be initiated by matured parasitized red cells and cytokineswhich trigger off coagulation cascade. It was further observed thatPT and APPT revert to normal as test subjects become afebrile and aparasitemic. This finding was supported by the work of Smith *et al*(17). The prolonged PT time was suggested by Jayashankar and colleagues to be caused by possible reduction in the plasma level of one or more of the factors in the extrinsic coagulation pathway (Factor VII, X, or V, prothrombin or fibrinogen) below about 30% of normal. In the same vein, that of APTT time could be due to a drop in plasma level of one or more of the coagulation factors other than factor VII to levels below about 30% of normal (4).

Conclusion

Coagulation mechanism is altered in malaria and the degree of alteration is proportional to the parasite density, this could precipitate bleeding in severe cases.

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