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Intracellular Quantification of Haemozoin Concentrations and Disease Severity of Malaria in Nigerian Children

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ABSTRACT

Severe falciparum malaria remains a leading cause of mortality, especially among children in the first five years of life. The possible role of parasite factors or genotypes as virulence traits in the pathogenesis of the severe disease has not been clearly defined. Haemozoin production has been implicated as a parasite marker of severe malaria. We have compared haemozoin production in *Plasmodium falciparum* isolates of children presenting with severe malaria versus those presenting with acute uncomplicated malaria in Ibadan, Nigeria. The haemozoin measurement was done by spectrometric measurement of ferriprotoporphyrin incorporated into a α-haematin. The mean (geometric) concentrations of Ferriprotoporphyrin (FP), was significantly higher in the severe malaria group (19.3mm/L) than in the uncomplicated group (18.2mm/L), $p<0.001$. Further analysis showed that parasite density ($\beta=-0.006$, $p=0.01$) and disease severity ($\beta=0.0029$, $p<0.001$) predict the concentrations of FP ($r^2=0.41$). These findings show that haemozoin production in severe malaria isolates may contribute to the survival and virulence of these parasite types in the host, thus influencing severity. We conclude that haemozoin production is a parasite feature that is strongly correlated with clinical severity of malaria.

Keywords: Plasmodium falciparum, isolates, haemozoin, Ferriprotoporphyrin IV, virulence

INTRODUCTION

Falciparum malaria remains one of the leading causes of morbidity and mortality in many tropical regions of the world, especially in Africa and Asia. While malaria itself has been known for centuries, the pathophysiology of severe disease is only being slowly unraveled. Thus, it is only in the recent past that parasite-host interactions associated with disease severity have been identified, including cytoadherence, rosette-formation and haemozoin production (Berendt *et al* 1989, Carlsson *et al* 1990, Metzger *et al* 1995)

Malaria pigment or Haemozoin is produced as the end product of haemoglobin digestion, a vital process for the intraerythrocytic development of the malaria parasite. During the intra-erythrocytic stage of malaria, free heme, which is extremely hazardous to the cell membrane, is released inside the malarial compartment. The malarial parasite detoxifies heme either through the mechanism of heme polymerization (Francis *et al* 1997), or degradation of heme by H_20_2 in food vacuoles (Loria *et al* 1999, Papalexis *et al* 2001) or by reduced glutathione in the parasite cytoplasm (Ginsburg *et al* 1998, Platel *et al* 1999). Nguyen *et al* (2003) also suggested an important role for PfHRP2 in the neutralization of toxic heme in the parasite cytoplasm.

Haemozoin is formed as the remaining toxic heme

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forms a crystalline dimer of α-haemtin complexed. Haemozoin crystals closely resemble β-haematin. Biochemically, haemozoin is composed of a high percentage of proteins, carbohydrates, trace amounts of lipids and nucleic acids and of the iron porphyrin, ferriprotoporphyrin IX (FP), the toxic heme (Goldie *et al* 1990, Pagola *et al* 2000). FP polymerizes into, the principal pigment of haemozoin, with the aid of a heme polymerase and PfHRP2 (Slater and Cerami 1992, Huy *et al* 2003). When the parasites are liberated at schizogony, Haemozoin is also released into the bloodstream.

The presence of haemozoin-containing leukocytes in the circulation is an indicator of the presence of Plasmodium spp. However, pigmented monocytes remain in the circulation for a prolonged period of time and may also indicate a recent, successfully treated malaria episode (Metzger *et al* 1995, Day *et al* 1996). Haemozoin production has been implicated in the severity of malaria. Orjih and Fitch (1993) found that haemozoin production differed between cultured laboratory lines of chloroquine sensitive and resistant strains of *P. falciparum*. Determination of haemozoin-containing leukocytes may serve as a prognostic marker for disease severity and progression. Several studies showed that intraleucocytic haemozoin correlated with prognosis of malaria infection among adults (Nguyen Hoan Phu *et al* 1995) and with severity of clinical infection among children (Amodu *et al* 1998, Luty *et al* 2000, Hanschield *et al* 2008, Grande & Boshetti 2011).

In the present study, we investigated the association between intracellular haemozoin production as estimated by the biochemical quantification of ferriprotoporphyrin IX incorporation into β-haematin (FP-β) in isolates of *P. falciparum* from children with the acute uncomplicated malaria and the severe disease.

MATERIALS AND METHODS

The study was carried out at the University College Hospital, Ibadan, in southwest Nigeria. The study site, a holoendemic area for malaria, is characterized by intense malaria transmission. Ethical approval was obtained from the hospital ethical committee and informed consent from the mother of each child studied.

Each child had a thick peripheral blood smear stained with Giemsa and examined for malaria parasites. Parasite density was estimated using standard methods. Haematocrit (packed cell volume) estimation was also done for each child.

Blood samples for the study were obtained from two groups of children presenting to the Children Emergency ward and Children Out-Patient clinic of the University College Hospital, Ibadan, with acute falciparum malaria were recruited into the study. The first group consisted of 29 children presenting with acute uncomplicated malaria group while the second consisted of 35 children presenting with severe malaria as defined by the World Health Organization (WHO 2000). Two milliliters of blood were collected from each child under aseptic conditions for the quantification of FP-β.

The method of quantification of Haemozoin was a modification of the method by Orjih and Fitch (1993). The samples were centrifuged at 2,000 r.p.m for 30 minutes and the supernatant fluid was discarded. Five milliliters of cold sodium phosphate (pH 7.6) was added to haemolyse the cells, shaking the solution vigorously. The sample was kept on ice for 10 minutes before centrifuging again for 30 minutes. The supernatant was discarded and the brown pellet (which contains Haemozoin) was washed by suspending it in 10mls of 20mM Tris buffered solution (pH 7.2). This was again centrifuged for 30 minutes at 2500 r.p.m, the supernatant was discarded and the pellet was resuspended in 2mls of a solution of 2.5% SDS and 25mM Tris (pH 7.8). This was left overnight or for a period of 16 hours at room temperature.

The insoluble material from the 2.5% SDS suspension was pelleted out by centrifuging at 2500 r.p.m, for one hour. The supernatant fluid was discarded and replaced with 1.8mls of freshly prepared 2.5% SDS/Tris buffered solution and 0.2mls of 1N NaOH to achieve a concentration of 0.1N NaOH. This was left for two hours at room temperature for the hydrolysis of β-haematin. The FP was measured with the aid of a spectrophotometer and read at wavelength 400 nanometers (absorption spectrum of FP) for each sample.

The final calculation of the FP concentration was based on the number of erythrocytes per 10^6 and the percentage of parasitaemia and the volume of the solution used as described by Orjih and Fitch (1993). FP-β concentrations and FP-β concentrations adjusted for haematocrit were calculated for each sample.

Statistics was done with the SPSS 9.0 statistical package (SPSS Inc., Chicago, USA). Exploratory data analysis showed parasite density and FP concentrations had skewed transformations. Log transformation of these variables was therefore carried out before analysis. Comparison of the two groups for log parasite density, haematocrit, log FP and adjusted log FP were done using Student's t test for independent samples. Pearson's correlation coefficients were used to assess correlations. Linear regression used to assess predictors of adjusted log FP values from among log parasite density and

severity of malaria (coded 1=severe, 0=uncomplicated). Statistical significance was set at p<0.05.

RESULTS

The geometric mean parasite density for the severe malaria group 15,083/μL, was significantly higher than the uncomplicated malaria group, 4,032/μL, p<0.001 (see Table 1). The mean packed cell volume for the acute uncomplicated malaria group was 29.8% while the severe group was 19.5%, p<0.001.

. The geometric mean concentration of FP-β in the acute uncomplicated malaria group was 14.4mmol/L compared 18.2, p=0.297 in the severe group. However the haematocrit adjusted FP-β values was significantly higher in the severe malaria group compared with the acute uncomplicated malaria group (18.2 mmol/L vs with 19.5mmol/L, p<0.001).

In the Severe malaria group, log parasite density was strongly correlated with haematocrit (correlation coefficient 0.59; p=0.003) but there was no such correlation in the acute uncomplicated malaria group. A regression model was constructed to determine if the severity of malaria and the parasite density could successfully predict the concentrations of FP-β. Table 2 shows that the log parasite density (β= -0.006, p= 0.01) and the severity of the disease (β=0.0029, p<0.001), can successfully predict the concentrations of FP with adjusted r^2 = 0.41, (p=0.005), that is, the higher the concentrations of FP-β, the more severe the form of malaria expected.

Table 1:
Quantification of Ferriprotoporphyrin In two Groups of Children with Malaria

	UM	SM	**t**	**p**
No. of isolates	29	35		
*Mean Parasite density[a] per μL	4032	15083	-2.374	<0.001
*Mean haematocrit (%)	29.8	19.5	5.242	<0.001
*Mean log FP adjusted for haematocrit	18.2	19.4	-4.989	<0.001

**Geometric mean*

Table 2: Regression Table
Outcome variable: Log FP corrected for haematocrit

Variable	β	SE (β)	t	p
Log parasite density	0.006	0.003	-2.478	0.017*
Severity	0.029	0.005	5.883	<0.001*
[constant]	1.283	0.009	135.409	<0.001*

adjusted r^2 =0.41; severity =1, uncomplicated =0
*p = 0.05

DISCUSSION

Pathological alterations accompanying falciparum malaria coincide with the release of haemozoin into the peripheral blood at schizogony. Haemozoin accumulates in the phagocytic and endothelial cells of the host after schizogony (Metzger *et al* 1995, Day *et al* 1996, Whitten *et al* 2011). Previous studies have demonstrated some association between the presence of pigment in leucocytes and severe malaria (Metzger *et al* 1995, Amodu *et al* 1998). Haemozoin has also been used as a prognostic indicator of total parasite burden in severe malaria particularly with "smear-negative" cerebral malaria cases and malaria associated severe anaemia accompanied by low parasitaemia (Metzger *et al* 1995, Nguyen Hoan Phu *et al* 1995, Awandare *et al* 2007, Kremsner *et al* 2009).

Haemozoin has been found to induce the activation of circulating monocytes to increase the release of cytokines such as TNFα, which upregulate the expression of endothelial cell surface adhesion molecules promoting vascular pathology and consequently tissue damage. In severe malaria, high plasma concentrations of TNFα correlate strongly with fatal outcome (Kwiatkowski *et al* 1990, Pichyangkul *et al* 1997, Singh *et al* 2000, Jain *et al* 2008).

Orjih and Fitch (1993) from their study of haemozoin concentrations in a chloroquine–resistant and chloroquine–sensitive strain showed that the haemozoin concentrations in the red blood cells containing chloroquine-resistant parasites were higher than chloroquine-susceptible parasites in the presence of Chloroquine (CQ) concentrations. They suggested that this might be due to an increased amount of heme polymerase activity with no reduction in haemoglobin degradation and consequently haemozoin production even after maximal inhibition of chloroquine or CQ may be ineffective against the heme polymerase of CQ--resistant forms of *P.falciparum*. Findings from this study, demonstrate an increase in haemozoin production

in severe malaria isolates compared with uncomplicated malaria isolates and this may be as a result of an increase in haemoglobin degradation and heme polymerization activity. This strongly indicates that the heme polymerase or some other features in the heme polymerization pathway of parasite isolates from patients with severe malaria may be significantly different from those with uncomplicated malaria. Thus studies on the heme polymerization / detoxification pathway may further elucidate these differences.

Results from this present study showed that intracellular haemozoin production is significantly higher among the severe malaria isolates than in the acute uncomplicated malaria isolates. Haemozoin production was strongly correlated with disease severity even when some confounding factors such as the packed cell volume (PCV) were corrected for. These findings suggest that there is a pathway in the parasite, responsible for an increase in haemozoin production among the severe malaria isolates than in the uncomplicated malaria. Hemozoin and its constituents affect the function of host cells in various ways. Studies have shown that haemozoin induces production of inflammatory cytokines such as IL-1β and TNF-α and inhibition of macrophage function (Sherry *et al* 1995, Taramelli *et al* 2000). In vitro studies demonstrated by Jaramillo *et al* 2003, 2004, also showed that synthetic haemozoin enhances IFN-γ-inducible nitric oxide synthase (iNOS) and the chemokines macrophage-inflammatory protein that may mediate enhanced migration of macrophages and neutrophils. These cytokines and chemokines have been shown to inhibit erythropoiesis a strong feature of a severe malaria manifestation, severe malaria anaemia (Dufour *et al* 2003, Lamikanra *et al* 2009). Thus the higher levels of haemozoin in severe malaria isolates as demonstrated in this study, may influence the stimulation of an increase in the release of cytokines in the circulation, which may be responsible for the strong correlation with severe malaria.

In conclusion, we have shown from this study that haemozoin production is a parasite feature that is strongly correlated with clinical severity of malaria. This suggests a strong role for this feature in the pathogenesis of severe malaria and may be a useful marker of virulence in malaria.

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