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COMPARING HAEMOZOIN COUNT AND PARASITAEMIA IN THE PROGNOSIS OF SEVERE *PLASMODIUM FALCIPARUM* MALARIA IN CHILDREN AND NON-IMMUNE ADULTS IN KANO-NIGERIA

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ABSTRACT
This study compared the value of pigment containing leucocytes (neutrophils and monocytes) counts and the malaria parasite density in the prognosis of severe malaria on four hundred and twenty (420) patients with clinical evidence of severe malaria in Kano-Nigeria. Three clinical groups comprising patients with impaired consciousness, patients with cerebral malaria and those with severe anaemia were identified. Samples were analysed for Malaria parasitemia and pigment count Giemsa's thick and Leishman's thin film respectively. Patients with impaired consciousness (n=217) recorded the highest malaria pigment count of 342.86 (±177.34) monocytes pigments/microliter. The highest parasite count of 234,962 (±264.5) per microliter was recorded among the cerebral malaria group. Patients with severe malaria and anaemia had the least neutrophils pigment and parasite counts of 219.0 (±140.96)/microliter and 212,232(±12.61)/microliter respectively. A linear relationship between the malaria parasite count and the Intraneutrophilic malaria pigment count in severe malaria was demonstrated. Pigment count proved a higher prognostic value in severe malaria compared to Parasitaemia.
Keywords: Haemozoin, Plasmodium falciparum, Parasitaemia, Malaria

INTRODUCTION

Haemozoin or Malaria Pigment is produced by malaria parasites during intraerythrocytic development as the end product of haemoglobin digestion after Schizogony (Slater *et al*., 1991). The pigment consists of a polymer of haem group linked by iron-carboxylate bonds (Slater *et al*., 1991). After its formation; the insoluble malaria pigment is eventually phagocytosed by scavenger neutrophils and monocytes and is easily seen inside these cells by light microscopy (White *et al*., 1991; White *et al*., 1995; Amodu *et al.,* 1998). Counting the number of pigments contained in peripheral blood phagocytes while examining the blood film for malaria parasites should give an indication of recent Schizogony in the deep vasculature and this could give a predictive value for malaria severity together with a prognostic value (White *et al*., 1995).

Parasite count has been used as a quantitative measure of infection and thus disease severity (Amodu *et al.,* 1998). However, the clinical value of this prognostic relationship in the diagnosis of individual patients is limited by its poor predictive value. Some patients are able to walk with 50% of their red cells parasitized, while others die with Parasitaemia <0.1 % (MacPherson *et al*., 1985). The pathology of severe falciparum malaria is related to the sequestration of red blood cells containing mature forms of the parasite in the microvasculature of vital organ; whereas only immature, less pathogenic ring forms are seen in the peripheral blood film (MacPherson *et al*., 1985). Thus the severity of malaria is mainly due to the sequestrated parasite though not readily seen by the microscopists (White *et al*., 1991). As the relationship between the circulating and sequestered parasite burden is very variable, depending on the stage and synchronicity of infection, the relationship between Parasitaemia and disease severity is therefore a loose one (White *et al*., 1991; White *et al*., 1995).

In *Plasmodium falciparum* infection, the presence of pigment in the large mononuclear and/or polymorphonuclear calls for long and thorough search for the parasite. A mononuclear cell response is a very useful clue that anaemia is due to malaria (Topley, 1998).

A mild malaria pigment together with marked mononuclear response and often nucleated red cells provide the classical picture of severe anaemia due to malaria (Topley, 1998). Previous studies have shown malaria pigment proved a better indicator of prognosis than peripheral parasite count (White *et al*., 1991; White *et al*., 1995). In tropical areas, where unsupervised use of antimalarial drugs is common, patients with illnesses consistent clinically with severe malaria but with negative blood smears pose a management dilemma (White, 1992). Microscopy for intraleucocytic pigment is valuable in the differential diagnosis of severe febrile illnesses in malaria endemic areas where uncontrolled use to antimalarial drugs is widespread (White, 1992). In areas of high stable malaria transmission where adults with malaria are asymptomatic (premonition), severe infections are thought to occur only in young children (White *et al*., 1991).

However, in a previous study, mortality was reported among 1.67% (1/60) of patients with severe malaria in Nigeria who had higher malaria pigment count in their leucocytes when compared with the survivors (Abubakar *et al.*, 2002). Similarly, mortality among 40/300 (13.3%) of adult patients with severe Malaria who had significantly higher proportions of malaria pigment – containing neutrophils on admission than the survivors were reported (Abubakar *et al*, 2002).

This work is aimed at comparing the malaria parasite density and the Intraleucocytic pigment count in the prognosis of severe *P. falciparum* malaria and disease severity among children and non-immune adults at Murtala Muhammad Specialist Hospital and Aminu Kano Teaching Hospital Kano Nigeria.

MATERIALS AND METHODS

Study Population:

Four hundred and twenty (420) patients with severe malaria and / or impaired consciousness and malaria parasite (*P. falciparum*) in their blood films were involved in the study. They comprised 70 adults and 350 children (among which 305 were males and 115 were females). The patients were recruited at the medical and pediatric wards of the Murtala Muhammad Specialist Hospital Kano, and Aminu Kano Teaching Hospital, Kano. Informed consent of the patients or relatives were obtained respectively before inclusion in the study.

Inclusion and Exclusion Criteria:

Patients with asexual form of *P. falciparum* in the blood film and any of the following features were eligible for the study:

i. Cerebral malaria with rousable or Unrousable coma.

ii. Severe (malaria) anaemia (Hb<5g/dl)

Patients who had received anti-malarials more than 12 hours before admission were excluded as well as those with no available Parasitaemia value after the first 2 treatment days. Evidence of morbidity or comorbidity with Cerebro Spinal Meningitis, hypoglycemia, and post ictal state were used to exclude patients from this study.

Study Design

The study is a cross-sectional study involving malaria cases with impaired consciousness and severe anemia as the sub group of patients with severe and complicated malaria reported within the study area and period. The subjects were sub-divided into three groups in accordance with Ejov *et al.* (1999) classification and grouped as follows:

Group 1: Patients with impaired consciousness: Glasgow score > 9/15, with or without other severe malaria complications such as circulatory collapse and shock, spontaneous bleeding, renal failure or sickle cell anaemia.

Group 2: Patients with cerebral malaria: Unrousable coma, Glasgow score < 9/15, with or without other severe malaria complications (such as pulmonary edema, circulatory collapse and shock, and fluid, electrolyte and acid-base disturbances).

Group 3: Patients with severe (normocytic normochromic) anaemia: Haemoglobin <5g/dl, in the presence of Parasitaemia, up to 10 x 10^3 per microliter and no other severe manifestations.

Specimen Collection:

Blood samples were collected into ethylene diamine tetra acetic acid (EDTA) containers on the first admission day of each patients or at least before the initiation of the antimalarial treatment. The blood was used for Packed cells Volume (PCV) estimation, haemoglobin estimation, total white cells count, film making for malaria parasite detection and parasite density estimation. Apart from film examination, all procedures were carried out within 2-3 hours of sample collection. The number of parasites counted per 100 leucocytes was multiplied by the total WBC of the patients to give a quantitative count per µL (White e*t al.*, 1991).

Pigment Estimation:

Haemozoin pigments were counted against 100 neutrophils and 30 monocytes and multiplied by the total WBC to express the pigments count per µL of blood.

Statistical Analysis: Statistical Analysis was done using Microsoft excel and Medcalc ^{R} statistical softwares to test for statistical significance of the difference in parasitaemia and pigment counts in the control and the three clinical group of patients.

Limitations: This study did not compare the three groups with mild or acute uncomplicated Malaria group (as additional control).

RESULTS

At the age of 1-10 years, it was observed that the ratio of male children with severe malaria is five times higher than that of the female (Table 1). We did not observe this relationship among the other age groups.

A linear relationship was demonstrated among the three clinical groups between the neutrophil pigments count and Parasitaemia (per microliter each) this did not apply to the monocyte pigments count. There was no significant difference in Parasitaemia and leucocyte pigments count between the impaired consciousness and the cerebral malaria group (P=3.488) (Tables 2 and 3).

A comparison in the mean Parasitaemia between these three groups has also revealed a significant difference between both the cerebral malaria and impaired consciousness groups and the severe anaemia respectively (P<0.001 and P<0.0001) (Table 5). Figures 1and2 show a Mann-Whitney's comparison in monocytic pigments count and neutrophils pigments counts among two clinical groups each. There was no significant difference in the monocytic pigments count between impaired consciousness and cerebral malaria groups using Mann-Whitney's U test (Figure 1). However, these two groups have shown significant difference from the severe anaemia group using two tailed student's t test (P<0.0001) (Table 3). The haemoglobin levels of all the three groups differ significantly from the control (P<0.001, P<0.001, P<0.0001) for groups 1 to 3 respectively (Table 4). A significant difference was observed in the Neutrophils pigments of impaired consciousness and severe anemia groups also using Mann-Whitney's U test (Figure 2).

Table 1: Age and Sex Distribution of the Patients with Severe Malaria in Kano Metropolis

Age group (years)	Male	Female
1-10	251	52
11-20	50	53
21-30	4	5
31-40	2	3
41-50	0	0

Table 2: Clinical, Parasitological and Hematological Features of Patients with Severe Malaria in Kano Metropolis

Group	CF	N	H(g/dl)	AP/µL	GC	Mean Neutrophil Pigments/µL	Mean Monocytes Pigments/µL
C	Apparently Healthy	60	13.0 (1.5)	0	0	0	0
1	Impaired Consciousness	77	*10.2 (0.8)	233,861 (342.3)	<9/15	291.22 (12.61)	342.86 (121.26)
2	Cerebral Malaria	126	*8.9 (0.7)	234,962 (264.5)	9/15	302.10 (177.34)	326.0 (120.33)
3	Severe Anaemia	217	*6.2 (0.2)	*212,232 (249.4)	0	*219.0 (140.96)	*269.0 (72.70)

Key: C = Control, N = Number, CL = Clinical features, AP = Admission Parasitaemia, H = Haemoglobin, GC = Glasgow Coma
Standard Deviation in Parenthesis, *Statistically significant difference (t-test)

Table 3: Students t-test for Significance of means of Leucocytic Malaria Pigment Count Among the Three Clinical Groups with Severe Malaria in Kano Metropolis

Clinical Groups Compared	Mean pigments difference (/µL)		95% CI of the difference		Test statistic t		Two tailed probability	
	N	M	N	M	N	M	N	M
Impaired Consciousness with Cerebral Malaria	10.8745	-1.3571	-38.109 to 59.9098	-50.7032 to 17.9889	0.437	-0.939	P = 0.6624	P = 0.3488
Impaired Consciousness with Severe Anaemia	-72.406	-73.5694	-110.7858 to -34.0261	-96.5214 to -50.6175	-3.713	-6.309	P = 0.0002	P < 0.0001
Cerebral Malaria with Severe Anaemia	-83.2804	-325.6336	-117.5295 to -49.0314	-341.6890 to -309.5782	-4.783	-39.894	P < 0.0001	P < 0.0001

Key: N = Neutrophilic, M = Monocytic

Table 4: Comparison of the Mean Haemoglobin Concentration of the Control Group with Three Clinical Groups with Severe Malaria in Kano Metropolis

Clinical Groups	Mean Haemoglobin difference (from control)	95% CI	Test statistic t	Level of Significance
Impaired Consciousness	-2.8	-3.19 to -2.41	-14.027	P<0.001
Cerebral Malaria	-4.1	-.42 to -3.78	-25.456	P<0.001
Severe Anaemia	-6.8	-7.01 to 6.59	-65.018	P<0.0001

Table 5: Comparison of the Mean Parasitaemia Among Three Clinical Groups with Severe Malaria

Clinical Groups Compared	Mean difference in Parasitaemia(/µL)	Standard error of mean	95% CI	Test statistic t	Level of Significance
Impaired consciousness with Cerebral Malaria	1,101	42.84	101.52 to 1185.48	25.97	P<0.001
Impaired Consciousness With Severe Anaemia	21,629	41.84	-21,701 to -21,556.81	-589.71*	P<0.001
Cerebral Malaria With Severe Anaemia	-22,730	28.57	-22,786 to -22,673	-795.721*	P<0.0001

*Statistically Significant

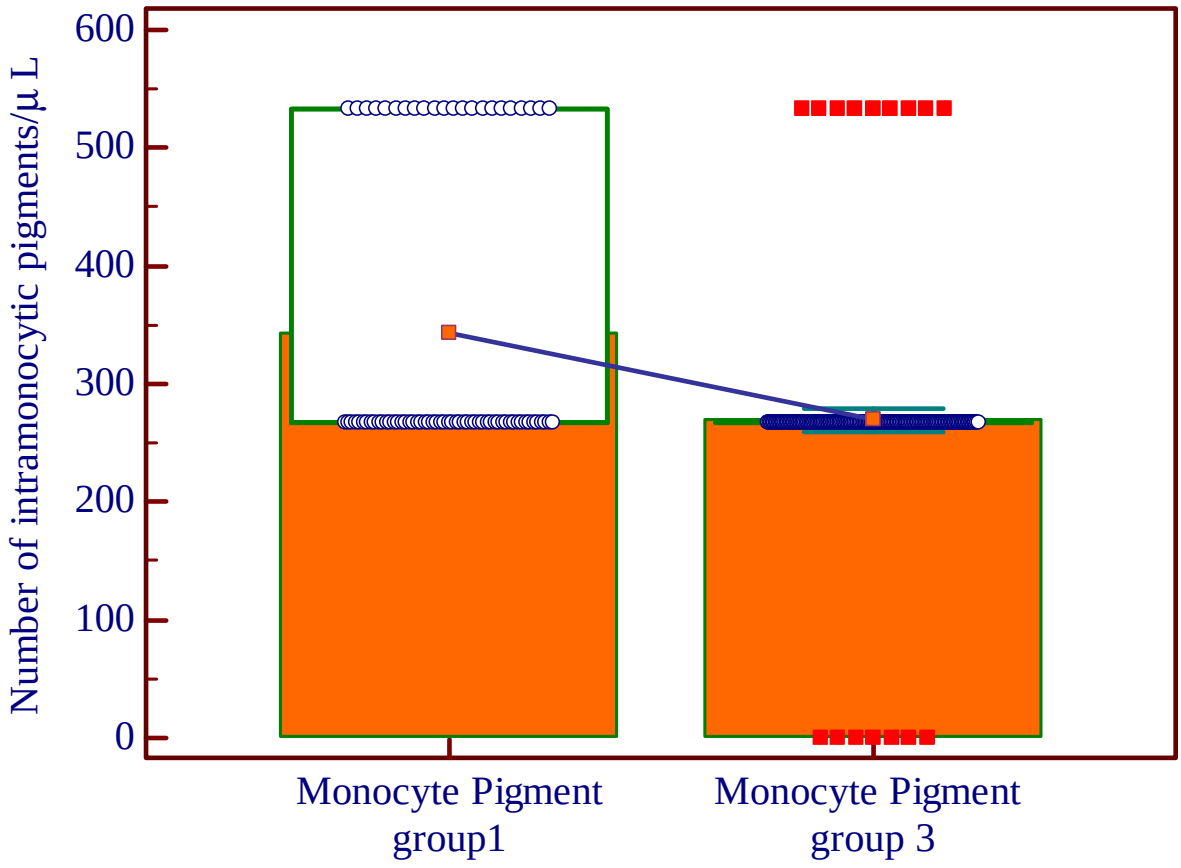

Figure 1: Mann-Whitney's Analysis of Monocytes Pigment Counts Between Impaired Consciousness and Severe Anaemia Groups

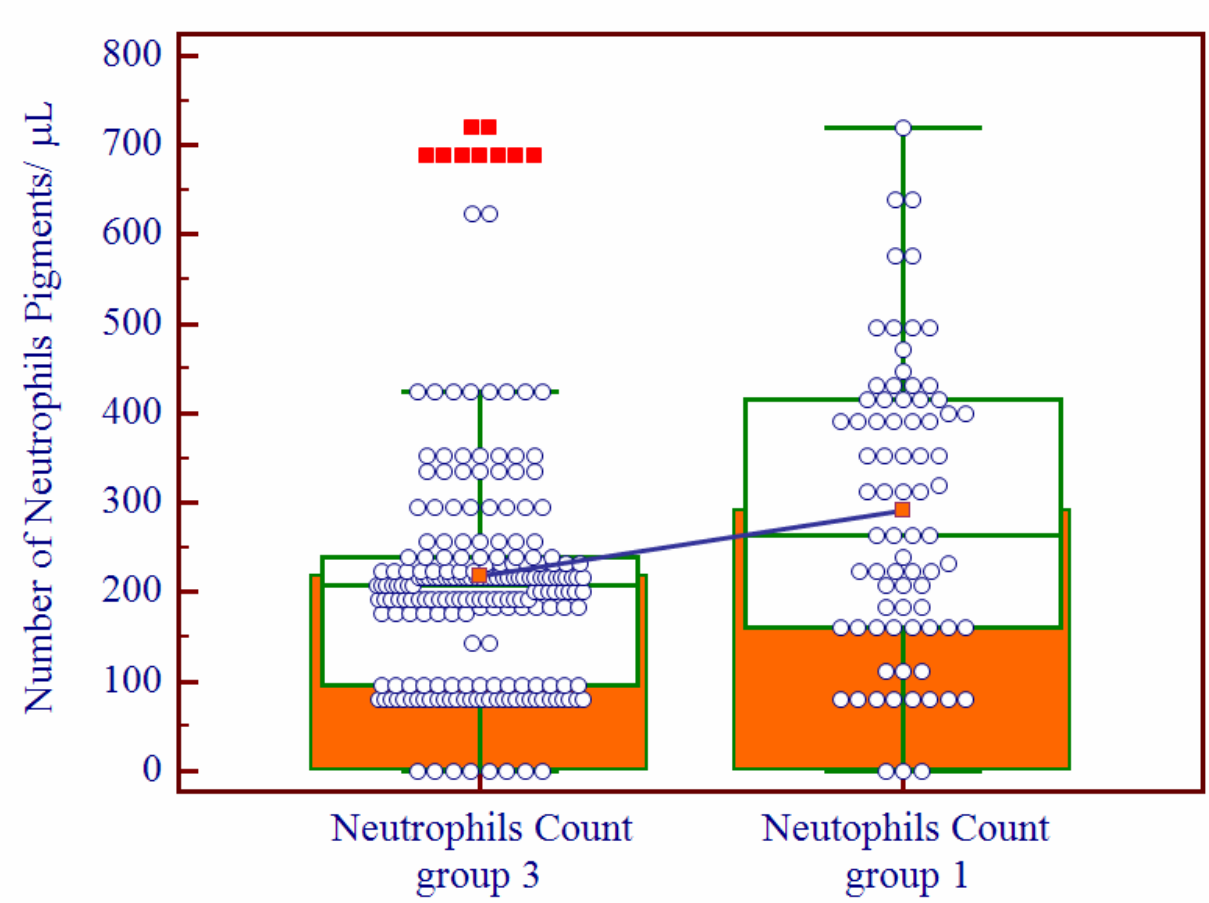

Figure 2: Mann-Whitney's Analysis of Neutrophils Counts Between Impaired Consciousness and Severe Anaemia Groups

DISCUSSION

Both parasite count and pigment – containing neutrophils counts are useful indicators for severity of malaria infection (Amodu *et al.*, 1998; White *et al.*, 1995; Abubakar *et al.*, 2002). However, the clinical value of parasite count in the diagnosis of individual patients is limited by its poor predictive value (White *et al.*, 1991). This study compared the value of pigment containing leucocytes (neutrophils and monocytes) counts and the malaria parasite density in the prognosis of severe malaria on 420 patients with clinical evidence of severe malaria. Among the three clinical groups studied, patients with impaired consciousness recorded the highest intraleucocytic (monocytic) pigment of 342.86 (121.26)/µL of blood. Patients with cerebral malaria and other complications (n=126) recorded the highest neutrophils pigment count of 302.10 (177.34)/ µL and the highest parasite count of 234,962 (264.5)/µL. Patients with severe malaria and anaemia had the least pigment count and parasite count of 219.09(±140.96) neutrophil pigments/ µL and a parasite count of 212,232 (249.4) /µL respectively. In all the three groups, a linear relationship between the malaria parasite count and the Intraneutrophilic malaria pigment count in severe malaria was demonstrated.

Slide examination (parasitaemia) has advantage over clinical examination and the data obtained from slides (for parasite density) tend to overestimate the malaria incidence in hyper- endemic area (Goverdhini *et al.,* 1991). Our findings are consistent with the two observations above. It is our belief that the malaria pigment count could serve as a good tool in the diagnosis, management and drug efficacy trials. Microscopy for both intraleucocytic pigments together with parasite count could be valuable in the differential diagnosis of severe malaria in area where uncontrolled use of anti-malaria drugs is widespread (Day *et al.*, 1996).

In a study on 146 Nigerian children with malaria, children with cerebral malaria were found to have the highest median value of 27.0% of pigment containing monocytes than children with mild (9.0%), asymptomatic (6.5%) or no malaria (20.%) (Amodu *et al.*, 1998). The study suggested that pigment containing monocytes and neutrophils count is a simple marker of disease severity in child hood malaria in addition to the parasite count (Amodu *et al*, 1998). The presence of malaria pigment in monocytes and neutrophils as a marker of disease severity has been validated previously. The association of pigmented neutrophils with cerebral malaria has also been demonstrated (Lyke *et al.*, 2003).

Our findings of a higher neutrophil pigments count in patients with severe malaria complications and those with cerebral malaria compared to those with severe anaemia corresponds to a previous reports in which peripheral parasitaemia and pigments were found to be strongly correlated with sequestered parasite loads on admission (Ochola *et al*., 2005). This is in line with our findings. It is evident from our work that both group 1 and 2 had high coma scores (9/15 each) with group 3 having lowest coma score (0/15). At same time group 1 and 2 recorded the higher pigment containing neutrophils count. The difference between group 1 and 2 is insignificant while the two groups differ significantly with group 3. This work has therefore strongly agreed with this findings (White *et al*., 1995; White *et al*., 1991; Abubakar *et al*., 2002).

Haemolytic anaemia with anisocytosis, poikilocytosis, polychromatia and hyper reticulocytosis has been observed. The anaemia of malaria is most common in children under 5 years of age, older patients with splenomegaly, pregnant women and people with sickle cell disease (Topley, 1998). The products of degranulation and the malaria pigment are toxic to host tissues and may be at least partly responsible for the induction of cerebral malaria (El-Shoura and Al-Amari, 1993). Although purified pigment is biologically inert, particulate malaria pigment derived from P. *falciparum* in culture has been shown to release large amounts of tumor necrosis factor (TNF) and interleukin IB (IL IB) (Pichiyangkol *et al*, 1994). In severe malaria, high plasma concentration of TNF have been shown to correlate strongly with fatal outcome (Kwiatkwoski and Greenwood, 1990). It is likely that some component of malaria parasite membrane, closely adherent to or absorbed on the pigment surface, is a malaria 'toxin' responsible for the cytokine inducing activity (Day *et al.,* 1996).

Particulate pigment could act as a potent vehicle of the delivery of toxin to cytokine producing phagocytes. The cytohedrant 'ghost' and pigment would act as persistent stimulus to local cytokine production in the microvasculature of vital organs (Day *et al.,* 1996). These ultra-structural observations may be relevant, to pathogenesis of coma, which takes place in some of patients with cerebral microvasculature and may explain the discrepancy commonly observed in light microscopy between the amount of apparently extracellular pigment in vessels and that in the peripheral blood (MacPherson *et al*., 1991). Two types of malaria pigment particles have been demonstrated in intraerythrocytic asexual forms (trophozoites & schizonts) while a single type was detected in gametocyte ultra-structurally (Pichiyangkol *et al*., 1994). Catabolism of host haemoglobin by malaria parasite liberates required amino acid precursors, but it also releases large amounts of potentially toxic haeme that accumulates in parasite food vacuoles during intra erythrocyte development (Eaton and Wood, 1993). The pigment – containing neutrophils count is a simple marker of disease severity in severe malaria in addition to parasite count and does not require special facilities (White *et al*., 1995; White *et al*., 1991; Abubakar *et al*., 2002). It is likely that the pigment containing neutrophil count would remain a relatively robust prognostic indicator in severe malaria at all levels of endemicity whereas monocytes may be less prognostically useful in areas of intense transmission (White *et al*., 1995). Findings of this study also suggests that the presence of the pigment in monocytes is a valuable marker of recent malaria in patients with negative films for malaria parasites either because of treatment or because the infection is self-resolving. Automated detection of malaria pigment using is therefore a useful malaria diagnostic tool in a semi-rural area. The test could be used in low risk malaria season for diagnosis because of its sensitivity. It can also be used in high risk malaria season for excluding malaria in suspected cases with negative pigment result because of its specificity (De Langen *et al*., 2006). Although this and previous studies had underscored the significance of malaria pigment count as a reliable indicator of tissue parasite load and disease severity, it should be appreciated that the sensitivity of pigment count may be attenuated by co-morbid tropical infections such as typhoid fever and HIV/AIDS that are associated with neutropenia or pancytopenia, which may result in spuriously low pigment count. Hence, there is the need to interpret pigment count results with caution in such cases (Abdool Gaffar *et al*., 1992; Bain, 1997).

Findings of this study suggest that pigment containing Leucocytes count could be a good prognostic tool in the measure of malaria disease severity. It would be found useful in clinical trials for the efficacy of antimalarial in addition as an alternative to parasite density estimation in severe malaria.

Recommendations

Hospitals and health care institutions in malaria endemic zones should endeavor to include pigment detecting haematology auto-analyzer in order to facilitate early identification of potentially severe malaria infection. And the clinicians, whom are traditionally only contented with the parasite count to diagnosis and assess severity of malaria, should be educated on the need to appreciate the significance of the pigment count as a more reliable indicator of disease severity within the context of uncontrolled use of anti-malarial drugs.

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