

Association of leucocytosis and hemozoin pigment in leucocytes with disease severity of malaria in children

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Abstract: Background: Malaria is the most important mosquito borne disease in humans and has been a major health problem worldwide. Detection of parasites by conventional microscopy is considered gold standard for diagnosis but low parasitemic conditions exhibiting only hemozoin pigment remains a challenge. Peripheral parasite density of *Plasmodium falciparum* is used as an indicator of disease severity, but does not quantify central sequestration. Detection of hemozoin pigment within parasite and the cytoplasm of phagocytic cells by microscopy is considered an important tool for the diagnosis of Malaria and its severity.

Objective: To assess the value of total leucocytes count in detecting a case of severe Malaria and to study the association of hemozoin containing neutrophils and monocytes with disease severity.

Method: A total of 152 children diagnosed with Malaria by peripheral smear or rapid diagnostic test admitted in the paediatric ward in Niloufer hospital, Hyderabad were taken for the study. Thin smears were prepared, stained with Giemsa stain, analysed for total leucocytes and differential counts and Malaria pigment. The quantity of polymorphoneutrophils and monocytes containing hemozoin pigment was determined. Results were compared at the end of the study.

Results: Out of a total of 152 Malaria cases, 49 were complicated and 103 were uncomplicated. Only 3 out of 49 complicated cases and 7 out of 103 uncomplicated cases were associated with leucocytosis which is not significant. Pigment was detected in 41 (83.6%) complicated cases and in 56 (54.3%) uncomplicated cases which is statistically significant. Pigment containing neutrophils were positive in 38 cases out of 41 complicated cases and in 41 out of 56 uncomplicated cases which is statistically significant. Pigment containing monocytes were detected in 36 out of 41 complicated cases and in 47 cases out of 56 uncomplicated cases which is statistically not significant.

Conclusion: There is no significant association of variations in total leucocyte counts with disease severity. Malaria pigment and number of pigment containing neutrophils were significantly associated with severe Malaria as opposed to uncomplicated Malaria. No significant association between pigment containing monocytes and disease severity was found.

Key Words: severe malaria, leucocytosis, hemozoin pigment

I. Introduction

Malaria is the most important mosquito borne protozoal disease in humans. According to the latest estimates, released in December 2013, there were about 207 million cases of malaria in 2012 (with an uncertainty range of 135 million to 287 million) and an estimated 627 000 deaths (with an uncertainty range of 473 000 to 789 000).¹

Light microscopy using Giemsa stain is widely used for malaria diagnosis, but its sensitivity varies depending upon the expertise of the observer. In addition, low parasitemia conditions (Ex: chronic infection, early stage of disease and partially treated condition exhibiting only hemozoin pigment) remains a challenge.² However, microscopy has remained the gold standard against which all other tests have been evaluated.³

Peripheral parasite density of *Plasmodium falciparum* is used as an indicator of malaria disease severity, but does not quantify central sequestration, which is important in the pathogenesis of severe disease. Malaria pigment, recognizable within the cytoplasm of phagocytic cells by light microscopy may represent a peripheral marker for parasite biomass. Hemozoin, also known as malaria pigment, is a product of haemoglobin digestion by Plasmodia.⁴ The presence of pigment correlates with mortality of severe malaria in Asian adults from an area hypo endemic for disease and with disease severity in African children.^{5,6,7,8}

Some of the known prognostic factors on the peripheral blood film are-

1. Parasitaemia⁹

2. Increased percentage of pigment containing neutrophils⁸ and
3. High WBC count.¹⁰

As part of parasite erythrocytic invasion, hemoglobin is proteolyzed releasing toxic heme. To detoxify soluble heme, a novel breakdown product known as hemozoin is created intracellularly. This digestive end-product of haemoglobin is sequestered in the *P. falciparum* digestive vacuole within infected red blood cells and released into host circulation during schizogony. Once this insoluble polymer is released into host circulation, scavenger neutrophils and monocytes phagocytose the material. It is easily visible by light microscopy, appearing as a black, brown, or amber pigment or as a birefringent crystal under polarized light.¹¹ Given that the typical half-life of a neutrophil is 6–8 hours and that of a monocyte is several days, the quantity and distribution of engulfed pigment within these phagocytic cells may reflect the chronology of a patient's infection. Detection of hemozoin pigment within parasite and mononuclear cells is observed to be an important tool for the diagnosis of malaria and considered as one of the signs of severity.^{5,12}

Mild leucopenia has been described in uncomplicated malaria, but a neutrophil leucocytosis is an important abnormality in patients with severe falciparum malaria and is associated with a bad prognosis as per some researchers. TNF- α may be responsible for this leucocytosis, which may be associated with a complicating bacteraemia. The other common changes seen are monocytosis in population living in endemic areas.^{13,14,15,16,17}

Malaria is sometimes associated with mild to moderate atypical lymphocytosis, leucopenia, leucocytosis, eosinophilia, neutrophilia and monocytosis.^{14,15,16} Phagocytosis of malaria pigment by monocytes, macrophages and less frequently by neutrophils has been observed in peripheral blood and bone marrow of patients with malaria.^{13,14,15,16}

Here, we made an attempt to study total leucocyte count and hemozoin containing leucocyte count in malaria and its value in diagnosing a severe case and its association with disease severity, as methods of estimating malaria disease severity and prognosis may be useful in stratifying patients in the early stages of admission for prognostication and intensive management.

Aims

- To study the total leucocyte count and hemozoin containing leucocytes in children with malaria.
- To assess the value of total leucocyte count, in detecting a case of severe malaria.
- To study the association of hemozoin containing neutrophils and monocytes with disease severity

Patients And Methods

Study design: prospective observational study.

Setting: Paediatric tertiary Institute of a government teaching hospital in Hyderabad, India.

Participants: 152 children aged between 1-12 years consecutively admitted with malaria.

Study period: January 2013 to October 2014.

Inclusion Criteria:

Children who are admitted and confirmed with malaria by peripheral smear or rapid diagnostic test between 1-12 years of both sexes.

Exclusion Criteria:

Children with severe disease or coma due to other associated causes.

Children who are already being treated.

Children who were partially treated for malaria in previous two weeks.

II. Methodology:

Having obtained institutional ethical committee approval, all children admitted with fever without clinical evidence of other diseases like bronchopneumonia, Urinary tract infection were identified and both Peripheral Blood Smear and Rapid Diagnostic Test were performed. Children showing positivity of either test were recruited. Those who showed clinical or lab evidence of concurrent other diseases like typhoid, septicaemia, dengue and immunodeficiency were excluded. Informed consent was taken from parents/guardian. These patients were classified into complicated and uncomplicated malaria based on WHO case definitions after detailed history, clinical examination and relevant investigations which were recorded on a pre-structured proforma.

Before instituting anti malarial therapy, venous blood was drawn with aseptic precautions and collected into a sterile EDTA test tube, thick and thin smears were prepared and stained with Giemsa method of staining. Thin smears were analyzed for absolute WBC and differential counts determined manually by a Pathologist who was blinded to clinical presentation and outcome.

Assessment of pigment:

In our study, hemozoin characteristics were explored as it relates to severe malarial disease. Because of the broad range of peripheral WBC counts and the disparity between neutrophil and monocyte percentages on absolute differential assessment, we standardized hemozoin measurements among groups by assessing the total amount of malaria pigment / mm³. We first analyzed groups by quantifying the percentage of pigmented monocyte and polymorphonuclear cells. Malaria pigment was detected on thin films by counting 100 polymorphonuclear cells (PMNs) and 30 monocytes and determining the quantity of cells containing pigment. A ratio was then determined of total pigmented PMNs/100 or monocytes/30. To standardize total pigment burden across variable absolute WBCs and differential counts as determined from thin smears, total PMN and monocyte pigment per mm³ were calculated as follows:

Total pigmented PMN/mm³ = (number of pigmented PMNs/100) × (absolute WBCs) × (percent of PMNs).
 Total pigmented monocytes/mm³ = (number of pigmented monocytes /30) × (absolute WBCs) × (percent of monocytes).

Data Analysis

From the data recorded in master chart, outcomes were analyzed, using chi square test. The statistical package used was IBM SPSS version 20.0.

III. Results

Among the 152 number of total malaria cases, 103 (67.7%) were uncomplicated and 49 (32.2%).

Table 1: Age Distribution Of Cases:

Age in years	Uncomplicated	Complicated
1 – 4	38 (36.89%)	14 (28.5%)
4 – 9	46 (44.66%)	24 (48.9%)
10 – 12	19 (18.45%)	11 (22.4%)

Table 2: Clinical Presentation In Complicated Cases:

complication	Number	Percentage
Cerebral malaria	10	20.4
Severe anemia	18	36.7
Hyperparasitaemia	8	16.3
Other criteria	6	12.2
mixed	7	14.2

Table 3: Total Leukocyte Count In Both The Groups:

TLC	Complicated n =49	Uncomplicated n=103
<4000	12	13
4000 - 11000	34	83
>11000	3	7

Chi square = 3.407
 Degree of freedom = 2
 p value = 0.1820

Table 4: Pigment Assesement In Cases:

	Uncomplicated	Complicated
Pigment detected	56 (54.3%)	41 (83.6%)
Pigment not found	47	8
Total	103	49

Chi square = 12. 35;
 Degree of freedom = 1
 p value = 0.0004

Table 5: Pigment Containing Neutrophils (Pcn) In Both The Groups:

	Complicated	Uncomplicated
PCN positive	38	41
PCN negative	3	15
Total	41	56

Chi square = 5.936
 Degree of freedom = 1
 p value = 0.01

Table 6: Pigment Containing Monocytes (Pcm) In Both The Groups:

	Complicated	Uncomplicated
PCM positive	36	47
PCM negative	5	9
Total	41	56

Chi square = 0. 288

Degree of freedom = 1

p value = 0.5915

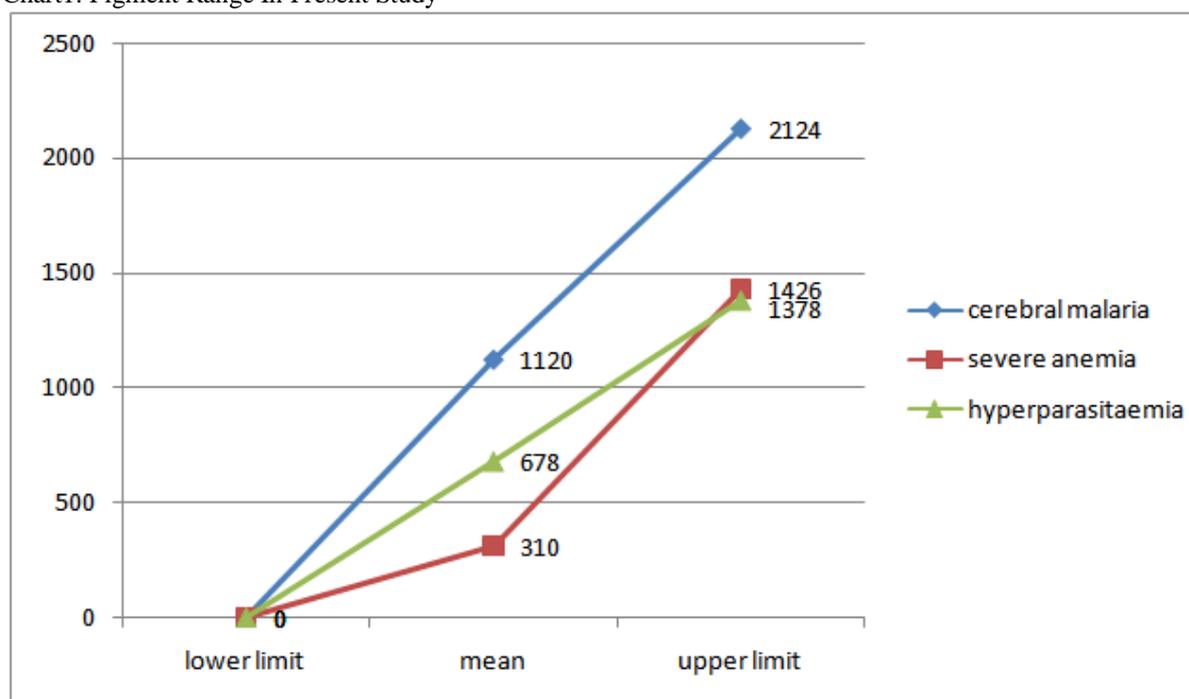
Table 7: Range Of Pigment In Both The Groups:

		Pigment containing neutrophils	Pigment containing monocytes
COMPLICATED MALARIA	Range	0 - 3624	0 - 909
	Mean	669	157
UNCOMPLICATED MALARIA	Range	0 - 1774	0 - 189
	Mean	193	69

Table 8: Range Of Pigment Association With Disease Severity In Complicated Malaria Group:

		PCNs
CEREBRAL MALARIA	Range	0 - 2142
	Mean	1120
SEVERE ANEMIA	Range	0 - 1426
	Mean	310
HYPERPARASITAEMIA	Range	0 - 1378
	Mean	678

Chart1: Pigment Range In Present Study



IV. Discussion

Malaria is associated with variations in haematological parameters. Conflicting results are reported about the total leukocyte count and its association with disease severity. As a part of the present study one of the objectives was to estimate this association between the complicated and uncomplicated cases of malaria.

Mild leucopenia has been described in uncomplicated malaras, but a neutrophil leucocytosis is an important abnormality in patients with severe falciparum malaria and is associated with a bad prognosis.

In 1995 Sowunmi A et al has studied 55 acute symptomatic falciparum positive African children and found that leucocytosis had statistically significant positive correlation in high parasitaemic (\geq or $>$ 10%) cases and found poor correlation in low parasitaemic cases ($<$ 10%).¹⁸

Iadhani s et al in 2002, found in their study that 20.1% of 1369 children with malaria had leucocytosis ($WCC > 16.5 \times 10^9/l$), in children over 3 months of age admitted in hospital. This leucocytosis was associated with prostration, coma, deep breathing, hyperparasitaemia, severe anaemia, and death in the univariate

analysis.¹³ In a study done on full blood counts in malaria by Thomas Hanscheid in the year 2003-2004, a total of 368 children were studied out of which 152 (88% were of > 1 year of age) had falciparum malaria. Children with severe malaria had slightly higher mean WBC values than children with non-severe malaria. The differences reached statistical significance only for the total WBC count and the neutrophil count in severe malaria cases as against uncomplicated malaria (p value of <0.05).²³

The above mentioned studies showed a statistically significant association of leucocytosis with severe malaria. In the present study 12 cases among the severe cases had leucopenia which accounts to 24.4%, 3 cases had leucocytosis 6.1% and the remaining 34 cases (69.3%) had leukocyte count between 4000 -11000. Total leukocyte counts between complicated and uncomplicated malaria did not reach a difference of statistical significance (p value = 0.1820).

Table9: Percentages of children with leucocytosis in severe malaria

Study	children with leucocytosis in severe malaria
Ladhani S et al ¹³	20.1%
Thomas H et al ²³	31.5%
Our study	6.1%

Changes in the WBC are less definite in malaria and there is a wide variation seen among the studies. In comparison to the above mentioned studies the present study's sample size was small and the method used for the estimation of total leukocyte counts was manual in contrast to instrument based estimation in above studies.

In the other studies they also included children of <1 year and hence this effect may even be more pronounced as children below one year and especially below three months tend to have rather higher WBC counts and higher lymphocytes and monocytes than older children. This explains the low leukocyte counts and the lack of correlation between leucocytosis and severity of disease in malaria.

Clinical evidence supports a role for hemozoin as an indicator for disease severity in both children and adults and for prognosis in adults. Additionally, malaria pigment itself may possess physiologic properties that contribute to the course of disease. Natural pigment has been demonstrated in vitro to induce production of both tumour necrosis factor and interleukin-1; this effect is ameliorated by protease digestion, suggesting the role of uncharacterized proteins.¹⁹ Monocytic cell dysfunction has been demonstrated (inhibition of oxidative burst, inability to digest hemozoin, or repeat phagocytic activity).²⁰

By analyzing the data as a calculated amount of pigment/mm³, a significantly higher amount of pigment for both neutrophils and monocytes was observed in the severe malaria group than the uncomplicated malaria group. These results validate the correlation of malaria pigment with disease severity (chart 1).

To illuminate differences between categories of severe malaria, subjects were stratified into one of the three predominant admission diagnoses; cerebral malaria, severe anemia and hyperparasitaemia (chart 1). A subset analysis was performed on children with the combined diagnosis of cerebral malaria with severe anaemia. Children with cerebral malaria had more pigmented PMNs/mm³ on admission.

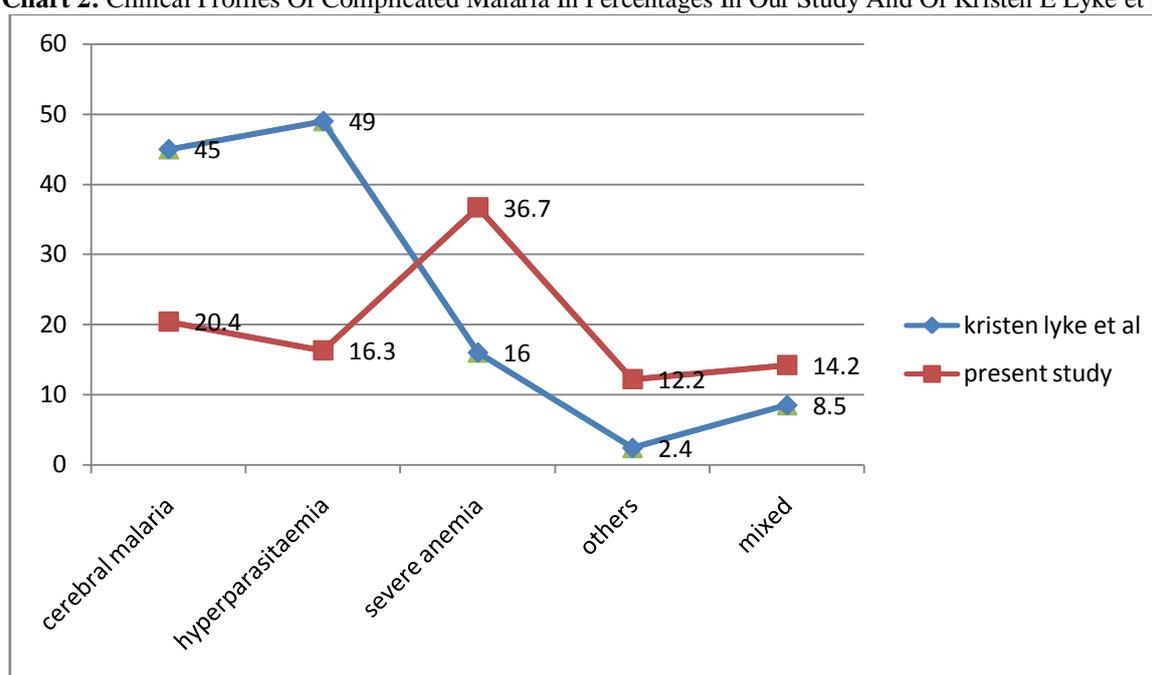
The proportion of neutrophils and monocytes containing malaria pigment is affected by total parasite burden and synchronicity of the parasite life cycle, and the clearance kinetics of these pigmented cells may be inherently different.

The results in the present study determined a statistically significant difference of pigment containing leukocytes in detecting a case of severe malaria. Even the range of pigment among the various manifestations of severe malaria was significant.

Among the total number of complicated cases, pigment containing leukocytes in the peripheral smear were found in 41 cases i.e. 83.6%. Whereas in uncomplicated cases only 56 (54.3%) displayed pigment containing leukocytes in the thin smears. This difference was statistically significant (p value of 0.0004).

In a case control study done in Mali, Africa, during 2000-2001 by Kristen E Lyke et al comprising of three groups each of 172 (severe, uncomplicated and healthy) children, a different clinical profile of complicated Malaria cases was observed as compared to our study.²¹ (chart 2)

Chart 2: Clinical Profiles Of Complicated Malaria In Percentages In Our Study And Of Kristen E Lyke et al.



The present study correlates with the study done by Lyke et al with respect to total pigment containing leukocytes and neutrophils to be associated with severity of malaria in comparison to uncomplicated cases. But there was no correlation with respect to pigment containing monocytes with a case of severe malaria in contrast to study by Lyke et al which proved a statistically significant difference of pigment containing monocytes among the severe and uncomplicated malaria.

In another study done in 1996 in Nigeria in 146 children aged 6 months to 14 years with cerebral, mild, asymptomatic and no malaria by Amodu OK et al, there was statistically significant difference in number of pigment containing neutrophils and there was no statistically significant difference in pigment containing monocytes in complicated versus uncomplicated Malaria which was similar to our study.⁸

Godfrey Mujuzi et al published in 2006 a study done in Northern Uganda comprising of 208 children aged between 6 to 59 months and showed a statistically significant association of pigment containing leukocytes with severity of malaria which correlates with our study.²² But in contrast the range of pigment containing monocytes was high in severe case group which was not observed in the present study.

Thomas Hanscheid et al in 2003-2004 has done a study on hemozoin pigment containing leukocytes in malaria, its diagnostic value and its association with disease severity.²³ In comparison with their results the present study also showed a significant difference of hemozoin containing neutrophils between the complicated and uncomplicated malaria groups. Similarly pigment containing monocytes did not show a statistically significant difference between complicated malaria and uncomplicated malaria.

Nzooza Munkwangu et al, in Zambia in 2008 studied on association of intraleukocytic malaria pigment with disease severity in 204 children with plasmodium falciparum malaria.²⁴ They were recruited into severe malaria (n=30), uncomplicated malaria (n=87) and control (n=87) categories.

The differences in the mean pigment-laden leukocytes in children with severe and children with uncomplicated malaria ($p < 0.05$) were statistically significant. Both pigment-laden monocytes and pigment laden neutrophils were significantly more in children with severe Malaria. When compared to this study, our study showed that pigment containing leucocytes and neutrophils only were significantly higher in severe malaria.

Their study observed a significant association between pigment-laden neutrophils ($p = 0.008$), lymphocytes ($p = 0.046$) and monocytes ($p = 0.043$) with severe anaemia. Coma ($p = 0.002$) and coma/severe anaemia ($p = 0.007$) were significantly associated only with pigment laden neutrophils. There was no significant association between high parasitemia and pigment-laden leukocytes.

In our study the range of pigment was more associated with cerebral malaria and hyperparasitaemia than with severe anemia (table 8).

Limitations Of The Study

- Inter observer variations in detecting the pigment containing leukocytes were not eliminated.
- The duration of illness was not taken into consideration in the study which has a bearing on the clearance kinetics of the pigment containing leukocytes.

V. Conclusions

- In the present study complicated malaria cases accounted to 32% of the total cases.
- Severe anaemia was the most common form of severe malaria in the study population.
- There is no significant association of variations in total leucocyte counts with disease severity.
- Malaria pigment is significantly associated with severe malaria as opposed to uncomplicated malaria.
- From present study, pigment containing neutrophils were significantly associated with severity of malaria.
- In the present study no association was found for pigment containing monocytes in determining the severity of disease

VI. Recommendations

- Present study recommends that while parasitaemia is important in diagnosis of malaria, the presence of pigment in leukocytes particularly neutrophils, be reported by microscopists alongside malaria parasite results and that this indicator may be considered as a basis to stratify patients for appropriate treatment and medical attention according to severity of the disease.

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