International Journal of Academic Health and Medical Research (IJAHMR)
ISSN: 2000-007X
Vol. 2 Issue 11, November – 2018, Pages: 13-18

Association of Intraleukocytic Malaria Pigment with Disease Severity, Diagnosis and Prognosis in Sudanese Patients

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***Abstract**: malaria is one of the most frequent hemoparasitic infections in tropical and sub-tropical countries. Malaria in Sudan is the major public health problem. This study was aimed to investigate the association of intraleukocytic pigment with malaria infection (severity, diagnosis and prognosis), and to investigate the correlation of parasite density levels with Malaria Severity. A total of 176 participants was drawn from the population of sudanese patient above 5 years, who attended or were admitted to the Bashayer Hospital, with diagnosis suggestive of malaria, they were selected for inclusion in the study. Blood films were examined first for malaria parasites diagnosis ,this was followed by detection of malaria pigment in both negative and positive films.Then Immunochromatography test was done, Subsequently haemoglobin concentration was determined. This study was approves that malaria pigment in peripheral blood leukocytes is evident for malaria disease , and makes the method of pigment determination appropriate and useful in malaria diagnosis, especially in patients with an illness consistent clinically with malaria but with negative blood smear due to haemolytic anaemia .A total of 176, 73 male and 103 female aged from 7 years to 69 years , drawn to Bashayer hospital with symptoms of malaria showed ICT positive result .Blood films were positive in 98(55.7%) and negative in 78 (44.3%) of patients . Malaria pigment was observed with the mean (22.07). There was an association between ICT and malaria pigment (P-value = 0.000).There was no association between BF and malaria pigment (P-value = 0.40).This study also approved that malaria pigment can be used in prognosis, were thirty five patient showed complications(19.9%) and 141 showed no complication(80.1%) ,there was association between complications and malaria pigment (P-value = 0.004) .This study also validates the presence of malaria pigment in leukocyte as a marker for disease severity, there was association between severity and malaria pigment (P-value = 0.02). While there is no association between density and severity (P-value = 0.980). So we conclued, intraleukocytic malaria pigment produced by parasites during intra erythrocytic development is associated with severe disease, mortality and it is a useful diagnostic indicator in anaemic patients with negative blood smears. The varied complexities of the current diagnostic methods make the method of pigment determination appropriate and useful. Secondly, malaria pigment is significantly associated with severe malaria. Thirdly, parasitaemia levels are neither associated nor correlated with malaria severity and therefore parasitaemia alone is not a reliable measure of malaria severity. We recommend the use of malaria pigment as marker for malaria disease severity. We recommend that parasitaemia should not be used alone as an indicator of malaria severity. We further recommend that, the presence of pigment in leukocytes to be adopted for diagnosis of malaria especially in cases of negative blood films, and recommend using malaria pigment as indicator for malaria prognosis.*

Keywords--- malaria; pigment;leukocytes

1. INTRODUCTION

Malaria is one of the most frequent hemoparasitic infections in tropical and sub-tropical countries, and it is most important parasitic disease worldwide. According to the latest estimates, released in December 2014 by WHO, there were about 198 million cases of malaria in 2013, with estimated 584 000 deaths .Most deaths occur among children living in Africa where a child dies every minute from malaria. Malaria in Sudan is the major public health problem. It leads to an estimated 7.5- 10 million cases and 35000 deaths every year(1).Malaria parasites belong to the genus *P*lasmodium (phylum Apicomplexa. In humans, malaria is caused by *P.* falciparum, *P.* vivax ، *P.*ovale and *P.*malariae .Among those infected, *P. falciparum* is the most common species identified followed by *P. vivax* . Although *P. falciparum* traditionally accounts for the majority of deaths, recent evidence suggests that *P. vivax* malaria is associated with potentially life-threatening conditions about as often as with a diagnosis of *falciparum* infection(2) .Mortality due malaria has been increase partly due to delayed identification of severe cases. Mortality due to malaria can be reduced by stratification of patients for appropriate treatment and medical attention. All this can only be achieved with correct diagnosis. Misdiagnosis can occur in area where the patient with an illness consistent clinically with severe malaria but with negative blood smear due to haemolytic anaemia ,malaria pigment or (haemozoni(H2)) the end product of the detoxification of haem in peripheral blood leukocytes (monocytes and granulocytes) is evident for malaria disease in greater than 90% of patients with malaria .

2. MATERILS AND METHODS

2.1 Study Design:

A prospective study design was used to assess intraleukocytic malaria pigment as it relates to malaria

severity, diagnosis and prognosis. This was a hospital based Cross sectional study. Conducted in Khartoum.

2.2 Study Plan:

Blood films for malaria were examined first for malaria parasites ,this was followed by detection of pigment in both negative and positive blood films .Then Immunochromatography test was done, Subsequently haemoglobin concentration was determined.

2.3 Study Area:

The study was conducted over a period of six months from November 2014 to April 2015 in Khartoum state At the Bashayer hospital.

2.4 Study Population and Sample Size:

A total of 176 participants was drawn from the population of sudanese patient above 5 years, who attended or were admitted to the Bashayer hospital, with diagnosis suggestive of malaria, , were selected for inclusion in the study.

2.5 Inclusion Criteria:

Patients above 5 years with *P*.falciprum malaria infection

2.6 Exclusion Criteria:

Patients below 5 years with or without malaria infection

2.7 Methods:

2.7.1 Stained Blood Films:

Thick and thin blood films for malaria were made on clean grease-free slides. Blood was obtained by finger pricking. The thin blood film was fixed with absolute methanol, and stained appropriately using Giemsa's staining method, these smears were stained with ten percent. Giemsa at pH 7.2 - 7.4 for 15 minutes. Giemsa stain is the most commonly used of the Romanowsky stains and is the best for routine diagnosis because of its applicability to both thick and thin smears, its stability on storage and its constant and reproducible staining quality over a wide range of temperatures . The stained films were then examined microscopically using 100 x objectives to estimating the parasite . One hundred microscope fields of the thick films were examined at l00x magnification before assigning a negative result.

2.7.2 Immunochromatography Test (ICT):

Immuno chromatography tests based on the capture of the parasite antigen from the peripheral blood using monoclonal anti-HRP-II antibodies and anti-aldolase antibodies against the parasite antigen targets was used. ABON PLUS was used

2.7.3 Determination of malaria Severity:

In this study, haemoglobin concentrations the main determinants of malaria severity.

2.7.4 Determination of Haemoglobin:

Haemoglobin concentrations in the patients were determined by the SYSMEX device.Venous blood was collected in EDTA tube.

2.7.5 Determination of Parasitemia:

Determinations of parasitemia were done using thick blood smears. Parasites density was estimated by crosses . Between 10 -100 fields (depending on parasitemia) were examined to determine the average number of trophozoites per thick film field. Ten fields are sufficient when the parasite density is high.

For designation of the relative parasite count on a thick film, a simple code of from one to four crosses or the plus sign is used to report parasite numbers:

\+ (1+) = 1 – 10 parasites per 100 thick film fields

\+ + (2+) = 11 – 100 parasites per 100 thick film fields

\+ + + (3+) = 1 – 10 parasites per one thick film field

\+ + + + (4+) = > 10 parasites per one thick film field

2.7.6 Determination of presence and quantities of the malaria pigment in leukocytes:

Was done using thin smears. Thin smears were fixed in absolute methanol for one minute before staining. These smears were then stained using Giemsa method for 15 minutes, then determined the white blood cell differential counts manually. Intraleukocytic malaria pigment was detected on thin films by counting 500 leukocytes and determining the proportions of haemozoin-containing neutrophils, lymphocytes and monocytes.

Total pigment-laden leukocytes per microlitre (3)were calculated as follows:

Total Pigment-Laden Leukocytes*Per Microlitre =

(Percent pigment-laden leukocytes* [in a count of 500WBCs])x (absolute WBC count per microlitre) X (percent leukocytes* in differential count of peripheral blood)

leukocytes* = neutrophils, lymphocytes or monocytes

2.8 Data Entry, Analysis and Presentation:

Microsoft Excel and SPSS were used to enter these data for analysis. Data is presented in the results as tables and figures.

3.RESULTS

3.1 Frequency Tables

3.1.1 Gender:

The study population according to gender was 73 males and 103 females .They were drawn to Bashayer hospital for malaria investigation or admitted for treatment. Table (1)

Table (1)

	Frequency	Percent	Percent	Cumulative Percent
Male	**73**	**41.5**	**41.5**	**41.5**
Female	**103**	**58.5**	**58.5**	**100**
Total	**176**	**100**	**100**	

3.1.2 (Age):

The study population according to age categories was from 7 years to 69 years with age mean (34.59), median (33), and mode (22) .Table (2)

Table (2)

	N	Mean	Median	Mode
Age	**176**	**34.59**	**33**	**22**

3.1.3 Malaria Pigmentation:

Malaria pigment was observed with the mean (22.07), median (17), and mode (10). Table (3)

Table (3)

	N	Mean	Median	Mode
Malaria Pigment	**176**	**22.07**	**17**	**10**

3.1.4 BF:

Out of 176 blood films examined for malaria parasites 98(55.7%) were positive and 78 (44.3%) were negative of patients. Table (4).

Table (4)

	Frequency	Percent	Percent	Cumulative Percent
Positive	**98**	**55.7**	**55.7**	**55.7**
Negative	**78**	**44.3**	**44.3**	**100**
Total	**176**	**100**	**100**	

- Chi square test for association between BF and Malaria Pigment (No association P-value = 0.40).

3.1.5 ICT:

All patients were positive by RDTS. Figure (1)

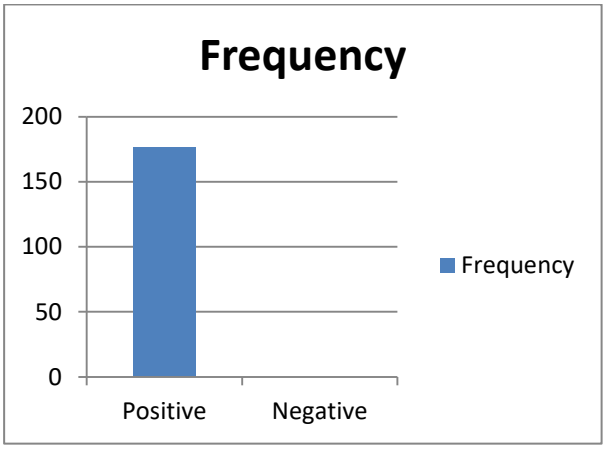

Figure (1): Frequency of positive results by ICT (RDTS)

- Chi square test for association ICT and Malaria Pigment (There is association P-value = 0.000).

3.1.6 Severity:

Disease severity among the malaria patients was, 38(21.6%) not sever, 138 (78.4%) with sever disease. Table (5)

Table (5)

	Frequency	percent	percent	Cumulative %
Not Severe	**38**	**21.6**	**21.6**	**21.6**
Severe	**138**	**78.4**	**78.4**	**100**
Total	**176**	**100**	**100**	

- Chi square test for association between Severity and Malaria Pigment (There is association P-value = 0. 02).

3.1.7 Complications:

Thirty five (19.9%) of the individuals were with malaria complications .Table (6).

Table (6)

	Frequency	Percent	Percent	Cumulative Percent
Positive	**35**	**19.9**	**19.9**	**19.9**
Negative	**141**	**80.1**	**80.1**	**100**
Total	**176**	**100**	**100**	

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- Chi square test for association between Complications and Malaria Pigment (There is association P-value = 0.004).

  - P-value more than 0.05 means there is No association.
  - P-value less than 0.05 means there is association.

3.1.8 Anaemia:
Anaemia was found In 111 (63.1%) of the Patients .Table (7).

Table (7)

	Frequency	Percent	Percent	Cumulative Percent
Positive	**111**	**63.1**	**63.1**	**63.1**
Negative	**65**	**36.9**	**36.9**	**100**
Total	**176**	**100**	**100**	

3.1.9 Density:
Among the positive malaria cases by microscopy the density was 43 were with one cross, 27 with 2 crosses, 23 with 3crosses and 27 with 4 crosses. Figure (2)

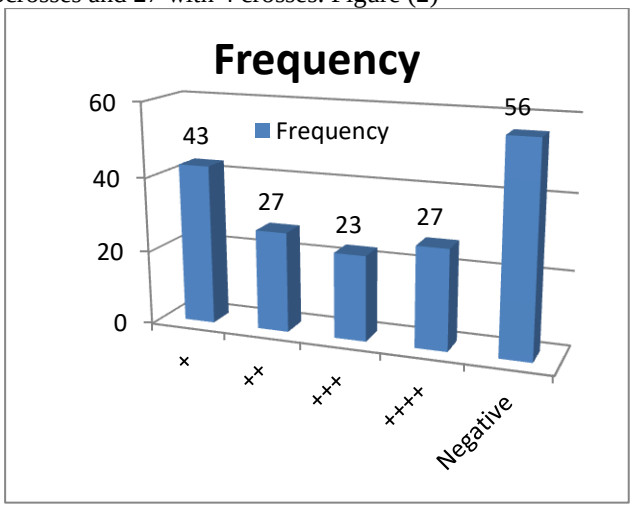

Figure (2): Density of parasitemia among positive cases by crosses

3.2 Cross Tabulation:
3.2.1 Density * Severity Cross tabulation :
No association between density and severity (P-value = 0.980) .Table (8)

Table (8)

			Severity		Total
			Severe	**Not Severe**	
Density	+	**Count**	**9**	**34**	**43**
		Expected Count	**9.3**	**33.7**	**43**
	++	**Count**	**5**	**22**	**27**
		Expected Count	**5.8**	**21.2**	**27**
	+++	**Count**	**5**	**18**	**23**
		Expected Count	**5**	**18**	**23**
	++++	**Count**	**7**	**20**	**27**

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Chi square test for association between Density and Severity (No association P-value = 0.980)

- P-value more than 0.05 means there is No association.
- P-value less than 0.05 means there is association.

3.2.2 Complications * Severity Cross tabulation:
There was association between Complications and Severity (P-value = 0.000) Table (9).

Table (9)

			Severity		Total
			Severe	Not Severe	
Complications	Positive	Count	22	13	35
		Expected Count	7.6	27.4	35
	Negative	Count	16	125	141
		Expected Count	30.4	110.6	141
Total		Count	38	138	176
		Expected Count	38	138	176

Chi square test for association between Complications and Severity (There is Association P-value = 0.000)

- P-value more than 0.05 means there is No association.
- P-value less than 0.05 means there is association.

4.DISCUSSION

A total of 176, 73 male and 103 female aged from 7 years to 69 years with age mean(34.59), median(33),mode(22).drawn to Bashayer hospital with symptoms of malaria showed ICT positive result .Blood films were positive in 98(55.7%) and negative in 78 (44.3%) of patients. Malaria pigment was observed with the mean (22.07). There was an association between ICT and Malaria Pigment (P-value = 0.000).There was no association between BF and Malaria Pigment (P-value = 0.40). This result approves that malaria pigment in peripheral blood leukocytes is evident for malaria disease and makes the method of pigment determination appropriate and useful in diagnosis especially in patients with an illness consistent clinically with malaria but with negative blood smear due to haemolytic anaemia . Our study result is similar to the result obtained by (Bojang (4). Who reported that malaria pigment has been shown to be a useful diagnostic indicator in anaemic patients with negative blood smears. Our study showed an association between malaria pigment and complications(haemolytic anemia, black water fever, coma) .Thirty five patient showed complications(19.9 percent) and 141 showed no complication(80.1 percent) ,There was association between Complications and Malaria Pigment (P-value = 0.004) .This result approved that malaria pigment can be used in prognosis. Our result agreed with (Kirsten E.Lyke et al(5))
finding who stated that total PMN pigment burden in patients with malaria was higher in those with cerebral manifestations and with combined cerebral manifestations and severe anemia , and that pigment was associated with a fatal outcome in patients with malaria., and demonstrates that pigmented leukocyte are associated with cerebral malaria and with death in patients with severe malaria .This our study showed an association between severity and complication , there was association between Complications and Severity (P-value = 0.000),this agreed with recent studies postulated that , the association of intraleukocytic malaria pigment with severity of malaria may facilitate the reduction of mortality from severe infection, and documented the association of severe and fatal malaria with the increased presence of malaria haemozoin in peripheral phagocytes. (Lell et al(6))
Our study showed an association between severity and malaria pigment, There was association between Severity and Malaria Pigment (P-value = 0. 02). While there is no association between density and severity, there was no association between Density and Severity

(No Association P-value = 0.980), this our result agreed with (Kirsten E.Lyke et al(5)).This study validates the presence of malaria pigment in leukocyte as a marker for disease severity. Malaria pigment, recognizable within the cytoplasm of phagocytic cells by light microscopy may represent a peripheral marker for parasite biomass.

5. Conclusion

The major conclusions that can be drawn from this study are:

Firstly, intraleukocytic malaria pigment produced by parasites during intra erythrocytic development is associated with severe disease, mortality and it a useful diagnostic indicator in anaemic patients with negative blood smears. . The varied complexities of the current diagnostic methods make the method of pigment determination appropriate and useful.

Secondly, malaria pigment is significantly associated with severe malaria.

Thirdly, parasitaemia levels are neither associated nor correlated with malaria severity and therefore parasitaemia alone is not a reliable measure of malaria severity.

Finally we can conclude that there is justification to reject the hypothesis for no association, since pigment-laden leukocyte, showed significant association with malaria severity, diagnosis and prognosis. Based on these results, the assessment of intraleukocytic pigment appears to be valuable for more detailed characterization of patients who present with *P*. falciparum clinical symptoms consistent with a malarial illness.

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