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Study on the Importance of Reticulocyte Count in Malaria Diagnosis at Kilifi County Referral Hospital

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Abstract: ***Malaria parasite infection is one of the leading cause of death in children and adults in this country the disease is more prevalent in the Nyanza and the Coastal regions of Kenya and has been reported that 30 - 50 percent of all out patients and 20% of all admissions in our health facilities are due to the disease. Due to its intercellular habitation which leads to infected cells being destroyed in the reticuloendothelial system leading to anaemia, a compensatory process by reticuloendothelial system to replace destroyed cells results in the haemopoetic sites to release reticulocytes. The aim of the study was to establish the relationship between malaria parasite infection and reticulocytosis in all children and adult populations diagnosed positive for malaria parasite by Giemsa staining between ages 3months and 45 years. The blood samples from sample population (2ml) were tested for normal or high values of reticulocytes by Methylene blue (supravital) staining methods and microscopic examination of thin blood films. The data was analyzed statistically by descriptive methods using measures of dispersion such as central tendency, frequency tables, bar charts and graphs and percentile to establish this relationship. In the study, it was found that severe malaria parasite infection leads to marked reticulocytosis. This phenomenon was observed more in children than in any other age groups and that according to sex, males were mostly affected than the females. Malaria parasite infection therefore conclusively leads to reticulocytosis across the sexes, age and the degree of parasitaemia determines the severity of reticulocytosis. Despite increased workload and the cost of doing this test, it should be included as a baseline test to all patients diagnosed with malaria infection in endemic areas. Frequent anti malaria campaigns should be carried out to minimize childhood deaths which may result to malaria complications. Research finding will be used to assist clinicians at Kilifi County Referral Hospital in management and monitoring of malaria.***

Keywords: Malaria Parasites, Reticulocytosis, Parasitaemia

1. Introduction

Malaria is one of the most common infectious disease and an enormous public health problem.It was discovered by Charles Louis Alphouse Laveran in 1880 as a black pigment in blood of people with malaria by the introduction of the any of the four Plasmodium species *(Plasmodium falciparum, Plasmodium ovale, and Plasmodium malariae and plasmodiumvivax*) into the human beings by infected female anopheles mosquito species which actively participate in the transmission.

Factors that determine the occurrences of malaria are those that influence the three components of the malaria life cycle, these are:

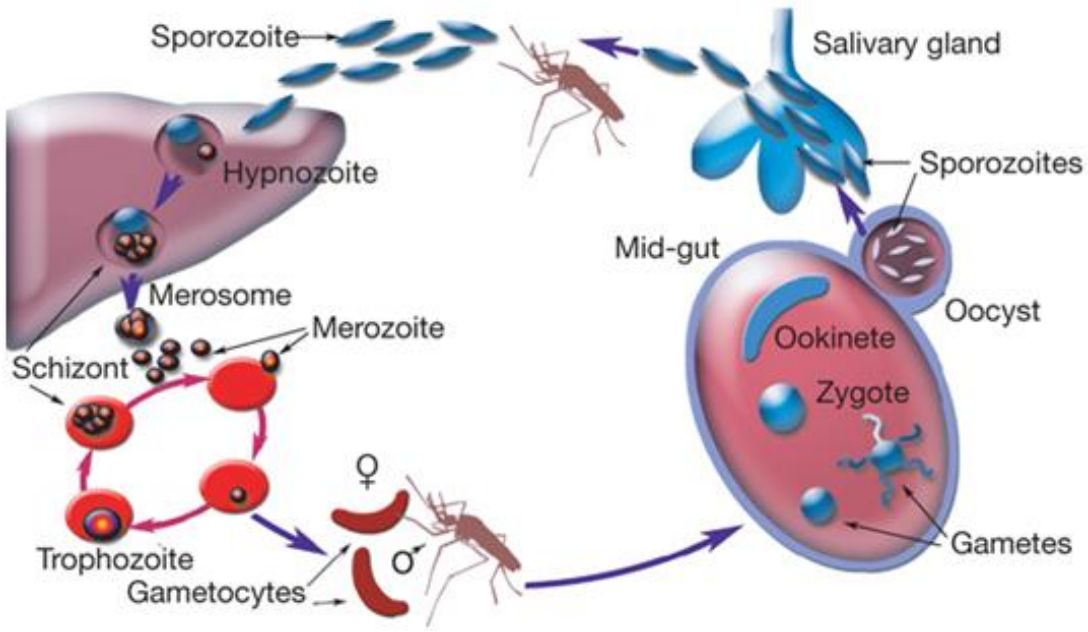
- Anopheles mosquitoes must be present which are in contact with humans and in which the parasite can complete the "invertebrate host" half of their life cycle.
- Humans must be present, who are in contact with anopheles mosquito and in whom the parasite can complete the "vertebrate host" half of their life cycle
- Malaria parasite must be present

Clinical features of malaria include **profuse sweating, Joint pains, headache, periodic cold chills.** In severe conditions malaria can lead to **coma, convulsions, anemia** and **renal failure**

Vertebrate Host Cycle: The malaria parasite life cycle involve two hosts. During blood meal malaria infected female anopheles mosquito inoculates sporozoites into the human host. The sporozoites infect liver cells and mature into schizonts which rupture and release merozoites. With *Plasmodiumvivax* and P*lasmodium ovale* a dormant stage hypnozoites can persist in the liver and cause relapses by invading the blood stream weeks or even years later). After this initial replication in the liver (exo-erythrocytic schizogony) the parasites undergoes asexual multiplication in the erythrocytes (erythrocytic schizogony).The merozoites infect red blood cells. The ring stage trophozoites mature into schizonts which rupture releasing merozoites, some parasites differentiate into sexual erythrocytic stage (gametocyte). Blood stage parasites are responsible for the clinical manifestation of the disease.

Invertebrate Host Cycle. The gametocytes, male (micro-gametocytes) and female (macro-gametocytes) are ingested by a female anopheles mosquito during a blood meal. While in the stomach of the mosquito, the micro-gametes penetrate the macrogamete generating zygotes. The zygote in turn become motile and elongated (ookinete) which invade the midgut wall of the mosquito where they develop into oocyts. The oocyts grow, rupture and release sporozoites which make their way into the mosquito salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle.

Malaria Parasite Life Cycle



Malaria Incidence: According to World Health Organization (Malaria Report 2021) the wide variation seen in the burden of malaria between different regions of the world is driven by several factors. First there's great variation in the parasite vector human transmission dynamics that favor or limit the transmission of Malaria and is most prevalent in Africa, South-East Asia and Western pacific. Studies also show that another factor contributing to regional and local variability in Malaria burden is different in levels of socio-economic development. Determinants include general poverty, quality of housing and access to health care and health education, as well as the existence of active malaria prevention and treatment access. The poorest nations generally have the least resources for adequate control effects. Reticulocytes are immature Red blood cells which develop and mature in the bone marrow and then circulate for about a day in the blood stream before developing into mature red blood cells. They are called reticulocytes because of a reticular (mesh-like) network of ribosomal RNA.A reticulocyte count in a blood test is performed to access the body's production of immature red blood cells. Anaemia being one of the complications caused by malaria, it is appropriate to calculate reticulocyte production index to understand whether the reticulocyte count is appropriate or inappropriate to the situation. Normal reticulocyte count in adults is 25 - 85×10/L (0.2 - 2.0 %), reference ranges. In anaemia, the reticulocyte percentage should be higher than "normal" if the bone marrow's ability to produce new blood cells remains intact..If the number of reticulocytes is not elevated in an anemic patient, there is increased likely hood that there will be some degree of bone marrow dysfunction.According to Hoffbrand A.V, Moss P.A.H, Petit J.E *(Dec.2019*) Essentials of Haematology 3rd Edition, some degree of haemolysis is seen in all types of malaria infections. The most severe abnormalities are found in *Plasmodium falciparum* infections. In the worst cases intravascular haemolysis is marked with haemoglobinuria. Patients with chronic malaria have anaemia of chronic disorders. Reticulocytes count rise in anaemia because of erythropoietin increase, and the severer the anaemia the higher the count.

Importance of reticulocyte count

- To evaluate severity of anaemia
- To provide information about the rate at which the bone marrow is producing red cells.

According to Cheesbrough M. (2010) District Laboratory Practice in Tropical Countries, Anaemia in falciparum malaria is mainly due to the destruction of parasitized red cells. Parasitized cells lose their deformability and are rapidly phagocytosed and destroyed in the spleen. The production of red cells in the bone marrow is also reduced and there is slow reticulocyte response.

Statement of the problem

In severe malaria infection affecting the population, excessive red blood cell destruction results in the hyperactivity of bone marrow and the reticuloendothelial system into releasing immature red blood cells-reticulocytes- in large numbers.

Broad Objective:

To determine the importance of reticulocyte count in malaria infection in patients attending Kilifi County Referral hospital.

Specific Objectives:

1) To show how malaria parasite density affects the degree of reticulocytosis.
2) To determine the age with high reticulocyte count in relation to malaria density.
3) To identify the sex with high reticulocyte count in malaria infection.

2. Methodology

2.1 Study area

Kilifi County Refferal Hospital, situated in Kilifi County in the Northern part of the Coast province, which is 65km from Mombasa city. The distance from Kilifi town to the hospital is I km. The hospital borders KEMRI/ Welcome Trust research laboratories. The land area in which the hospital is situated is about 5 acres.

2.2 Study design

Cross - sectional study using simple laboratory experiments because of the diversity of the population.

2.3 Study population

Patients attending Kilifi County Refferal Hospital laboratory. Qualification as study sample were all patients aged between 3 months and 45 years and who tested positive for malaria parasites by Giemsa staining from June to September 2021.Any patient who tests negative during the same period was excluded from the study.

2.4. Study variables

Parasitaemia, Gender and Age

2.5 Sample size

The sample size comprised of 195 patients derived by *Cochran W.G. (1977)* method.

$$n = \frac{Z^2pq}{d^2}$$

n= Sample size
Z= normal Standard 95%
P=Prevalence 15%
q= (1-p)
d=0.05 Level of significant error to be committed.
$n = (1.96)^2 \times 0.15 \times 0.85 / (0.05)^2$
=195patiens.

2.6 Sampling methods

Random sampling method by use of random tables was used. All the patients who had been tested for malaria and had tested positive were my sample population.

2.7 Material, Tools and Methods of Data Collection

2.7.1 Methods of data collection
Making thick and thin blood films

1) Clean, grease free slides were used.
   13 ul of patient blood collected in the Edta tube was placed to the right of the slide.
   Using the end of the plastic pipette, the blood was spread to make the thick film**.**
2) 10 ul of blood was placed at the center of the slide.
3) The drop was spread to make a thin film using smooth edged slide spreader at a steeper angle (45°).
4) A pencil was used to label each slide with the patient name, number and date.
5) The films were then air dried.
6) The films were fixed by flooding with 3 drops of absolute methanol and allowed to dry.

2.7.2 Procedure for staining

Cheesborough M (2010).Medical Laboratory Manual for Tropical Countries Page 193).

10% of Giemsa stain was prepared by measuring 45ml of buffered water (ph7.1-7.2) and 5g of Giemsa stain added and mixed gently.

a) Dried films were placed on a staining rack with the films facing upwards.
b) Using a Pasteur pipette, the films were flooded with the diluted stain for 15 minutes
c) The stain was washed off from the slide using clean tap water.
d) The back of the slide were wiped and the stained films placed on a drying rack.
e) A drop of oil immersion was placed onto a selected site on the thick film.
f) Counting of malaria parasites in the thick film per 200 white blood cells.
g) If the parasites were more than 25 per field in the thick film, it was examined again on the thin film and parasite count made per 500 red blood cells.
h) Confirmation of the plasmodium species was done by examining thin films that were prepared.

Results

Chromatin of the parasite -dark red.
Cytoplasm of malaria -blue.

Reticulocyte

2.7.3 Procedure for reticulocyte staining and counting

Kjeldsberg C.K, Practical Diagnosis of Hematologic Disorders- Fifth Edition, 2010.

a) 30 ul of New Methylene blue was delivered by means of automated pipette into a plastic tube,
b) 30 ul of patients EDTA blood was added and mixed gently.
c) The mixture was placed in a water bath at 37°C for 15 minutes for the cells to take up the stain.
d) Resuspension of the red cells by gentle mixing was done by gently shaking the mixture.
e) A drop of the mixture was placed on a clean slide.
f) Using a spreader, a thin film was made on a clean grease free slide by a forward rapid movement.
g) The preparation (film) was air dried on a rack.
h) A drop of oil immersion was placed on the film and examined using XI00 objectives for reticulocytes.
i) The number of reticulocytes was counted against 1,000 red blood cells.

3. Method of Analysis

Random sampling method by use of random tables was used. All the patients who had been tested for malaria and had tested positive were my sample population.

4. Data Summary and Analysis

Total number of response = 195

Table 1: Percentage frequency of malaria parasite infection against age groups

Age class (in years)	Frequency (f)	%	Midpoint
0-10	150	76.9	5
Nov-20	17	8.7	15.5
21-30	15	7.7	25.5
31-40	7	3.5	35.5
41-50	6	3.1	45.5
Total	195	100	

Mean age = $\frac{\sum FX}{\sum F}$ = 1917.5/195=9.833 ≃ 10 years

As can be determined from the curve on the next page:
Modal class =0-10 years
CvMode = ≃ 8 years
Median class = 0-10 years
Median = 8 years
SD = 10

Range = lowest age = 3 months

Highest age = 45years

Therefore range =45-0.25 = 44.75 years

a) 95% confidence intervals
Mean + 2(10)
10+2(10)
10+20=30
195+20=215
195-20=175

Total number of patients with malaria parasite infection was 195. Majority of patients with malaria were children below ten years and their frequency was 150(76.9 %). The mean age was 10 years. One hundred and seventy five patients were within 2SD interval of between 0-30 years. It was observed that children below 10 years had the highest number of frequency because they have not acquired enough immunity to protect them from diseases. The frequency is low in other classes, because most of these people have acquired immunity against the disease.

Table 2: Prevalence of reticulocyte level in malaria parasite infection per age group.

Age group in years	Frequency of total retic (both normal and elevated)	Frequency of reticulocytosis	Percentage prevalence of reticulocytosis
0-5 years (babies)	98	72	73.5
6-12 years (children)	28	22	78.6
13-18 years (adolescence)	15	10	66.7
19-45 years (Adult)	54	38	70.4
Total	195	142	72.8

Among 142 patients with reticulocytosis, children showed a higher prevalence of reticulocytosis 22 out of 28 (78.6 %.)

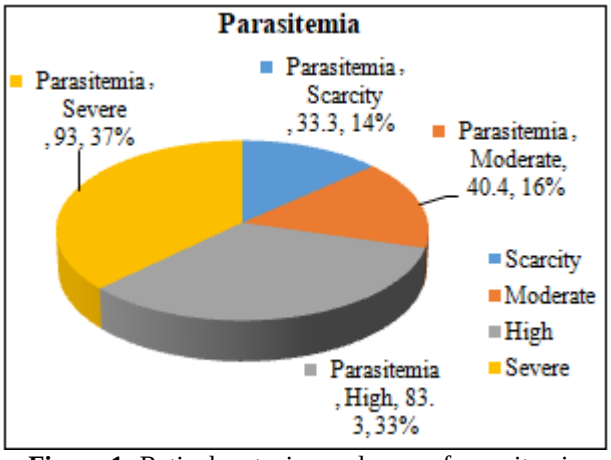
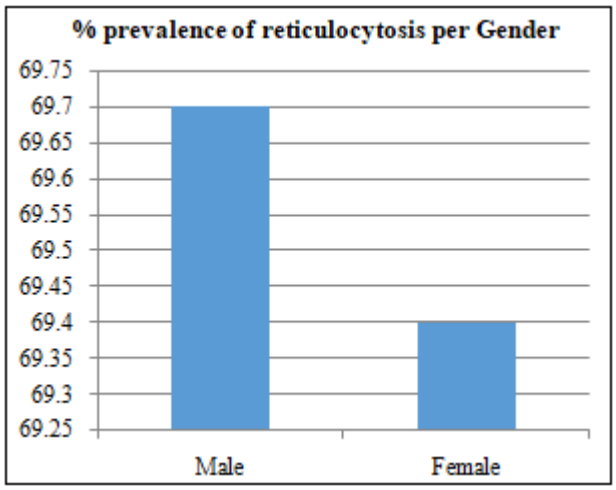


Figure 1: Reticulocytosis per degree of parasitemia

Among 195 patients with malaria parasite infection patients with severe parasitemia had a high prevalence of reticulocytosis 53 out of 57 representing 93.0%.



Among 126 children with reticulocytosis, male had a prevalence of 87.3% and female had a prevalence of 53.2%.and males showed a higher prevalence of reticulocytosis than females.

Hypothesis Testing

The Chi-square test f independence was used to test the hypothesis as follows:
H_0: Malaria and reticulocytosis are independent (no

association between the two).

H_1: Malaria leads to reticulocytosis (Malaria and reticulocytosis are dependent)

Significance level considered was 5%, since the data confidence was taken as 95%.

Therefore: $\alpha=5/100= 0.05$

Observed frequency (O) table:

Disease	Child	Adult	Total
Parastaemia	167	28	195
Reticulocytosis	94	48	142
TOTAL	261	76	337

Calculations of Expected data (E):

$\mathbf{E_{11}}$ = (261*195)/337 = 151.02
$\mathbf{E_{12}}$ = (76*195)/337 = 43.98
$\mathbf{E_{21}}$ = (261*142)/337 =109.98
$\mathbf{E_{22}}$ = (76*142)/337 = 32.02

Expected frequency table:

Disease	Child	Adult
Parasteamia	151.02	43.98
Reticulocytosis	109.98	32.02

$\mathbf{X^2_{.95Calculated}} = \sum(O-E)^2/E$

$= (167-151.02)^2/151.02+ (28-43.98)^2/43.98+ (94-109.98)^2/109.98+ (48-32.02)^2/32.02$

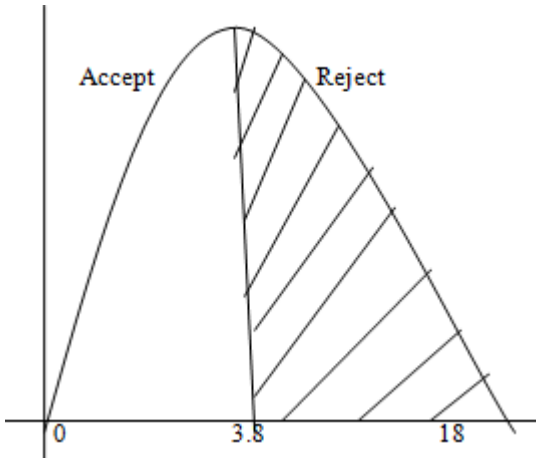
= 17.7941

$\mathbf{X^2_{.95Tabulated:}}$

V (degrees of freedom) = (r-1) (c-1)

= (1) (1) =1

Therefore $X^2_{Tabulated}$ =3.84



$X^2_{Calculated} > X^2_{Tabulated}$ i.e. 17.79> 3.84 hence the null hypothesis is rejected.

It is finally concluded that there is a significant association between malaria and Reticulocytosis.

5. Discussion

Malaria contributes significantly to anaemia. This occurs when parasitized red blood cells are rapidly destroyed in the spleen. The destruction of red blood cells causes the bone marrow to produce more red blood cells. if the bone marrow is not impaired then new young red blood cells will be produced hence reticulocyte count will be raised. In cases of bone marrow dysfunction reticulocyte count will be low because production of red blood cells will be impaired.

Anaemia is usual in malaria, it is often only mild or moderate but usually severe especially with falciparum infection. In this study, out of 195 respondents with malaria parasite infection, it was observed that one hundred forty two (72.8%) patients had reticulocytosis. Researchers who have reported reticulocytosis in chronic malaria include *Stephen et al* and *perrinet al (2019)*. In this study, fifty three patients (27.2%) had a normal reticulocyte count as shown in table 5).

According to age group, it was found that reticulocytosis was seventy two (73.5%) in babies, twenty two (78.6%) in children, ten (66.7%) in adolescents and thirty eight (70.4%) in adults giving a total 72.8 prevalence among 142 patients .In this study it was observed that patients who had high frequency of reticulocytosis were children. This disagrees with studies done at the North Coast of Papua New Guinea by *Oppenheimer et al (2019)* which showed that there is greater reticulocytosis in response to malaria infection babies which could be due to characteristics of area under study and also the timing of the study period while in the former it could be because babies may have not acquired enough immunity to fight malaria.

In adults out of a total of 69 patients with malaria ,forty eight(69.6%) had reticulocytosis and both sexes had about the same rate of reticulocytosis implying that the effect caused does not differ with sex, while twenty one (30.4%) had normal reticulocyte counts. In 126 children, ninety four (74.6%) had reticulocytosis and thirty two (25.4%) had normal reticulocyte count. From this data it's evident that in children, more patients suffer from reticulocytosis while in adults, majority had normal reticulocyte counts

According to sex among patients with reticulocytosis it was found that ninety two (64.8%) were male and fifty (35.2%) were female. This showed a higher frequency of male patients than female patients.

According to sex among children with reticulocytosis, it was found that sixty nine (87.3%) were males and twenty five (53.2%) were females. This indicates a higher frequency in male children than females. According to sex among adults with reticulocytosis, the difference was minimal. It was found that twenty three (69.7%) were males while twenty five (69.4%) were females.

According to parasitemia among patients with reticulocytosis, twenty three (40.4%) patients out of 57 had moderate parasitemia, sixty five (83.3%) out of 78 had high parasitemia, fifty three (93.0%) out of 57 had severe parasitemia. Patients with scarcity in parasitemia had low

prevalence of reticulocytosis one out of 3 patients (33.3%). Study done by *Saad H. Abdalla et al* (2018) also revealed that reticulocyte count was raised in chronic malaria (that is high and severe parasitemia. In this study it was found that reticulocyte count is raised in severe parasitemia. Scarcity in parasitemia did elevate reticulocyte count but by minimal ranges because the numbers of parasites are few to cause destruction of red blood cells since the few parasitized red cells will be removed by the spleen and the production of new red cells will not be triggered.

Therefore reticulocyte count is an important test in providing information about the rate at which the bone marrow is producing red cells and in evaluation the severity of anaemia caused by malaria parasite.

6. Conclusion

From the study that was carried out, it was observed that reticulocytosis is common in chronic malaria (that is high and severe parasitemia). It was also observed that reticulocytosis in relation to malaria parasite infection was frequent in children than other age groups because they have not acquired enough immunity and hence prone to malaria infection than those other age groups.

Reticulocyte count is a useful reflection of the erythroid activity in response to anaemia caused by malaria. Anaemia being one of the complications caused by malaria, it raises the reticulocyte count. Reticulocyte count is only elevated in anaemia if the bone marrow ability to produce new red cells remains intact. The degree of anaemia often correlates with the extent of the red cells parasitization and destruction of these cells in the spleen and a fall in haemoglobin concentration. In the presence of dyserthropoesis and ineffective erythropoiesis, the periphery reticulocyte count may be much lower reflecting bone marrow suppression.

It was therefore observed that malaria parasite infection affect production of reticulocyte and this depends on the severity of malaria infection and the ages.

6.1 Recommendations

- From the results obtained in the study, the recommendation is that despite the increase in workload and the cost of doing the reticulocyte count test, it should be included as a baseline test to all patients with malaria parasite infection especially in malaria endemic areas in order to assist in the assessment of severity of the malaria infection.
- The government should come up with frequent malaria campaigns so as to reduce the number of deaths in babies which occur as a result of severe anaemia due to malaria infection which eventually leads to reticulocytosis

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