Usefulness of histogram study in diagnosis of malaria: our experience at tertiary care center

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Abstract

Background: Malaria diagnosis presents a challenge to all laboratories. Especially post monsoon season, there is a need for rapid, sensitive and cost effective method to effectively screen all samples, especially when workload is very high. Automated cell counters offer rapid, sensitive and cost effective screening of all samples

Aims and objective: To evaluate the usefulness of automated cell counters analysing their histograms, scatter-grams and the flaggings generated in malaria positive cases. To compare other parameters which could help to identify malaria parasite in microscopic examination of peripheral smear.

Methods: The study was conducted in the laboratory of B. J. Medical College, civil hospital, Ahmedabad12 months of duration from November 2014 to November 2015. 775 samples from the patients who presented to emergency department and for whom the attending clinicians had requested full blood count and malaria smears were included in the study. Samples were processed as part of normal routine cell dyn ruby analyser for FBC (Full Blood Count). We had used histograms and scatterplots which were generated by cell dyn ruby and interpreted the histograms in malarial cases.

Result: Total 775 cases were studied, out of which 409 cases were positive for malaria which are confirmed by peripheral smear examination.409 positive cases were evaluated, abnormal scattergram were observed in 98% cases of malaria while abnormal WBC histogram peaks were noted in 92% cases of malaria. Haematological parameters such as anaemia, leucopenia, Monocytosis, atypical lymphocytosis, atypical eosinophilia, pink flag were also important for diagnosis.

Conclusion: This method of histogram study in malaria is a useful addition to conventional microscopy. Beneficial part of this study is that where workload is high and clinician send only CBC (complete blood count), in such circumstances histogram study in automated analysers as we have used cell dyn ruby in our institute, we can diagnose malaria. If we have trained staff for histogram and scattergram of automated analysers so we cannot miss sample in initial screening.

Keywords: Automation, histogram, Pink flag, WBC Scattergram, Malaria, atypical lymphocytes.

Introduction

Malaria is major health problem in tropics. In endemic areas especially in post monsoon season, it is not possible to manually screen all peripheral blood films for malarial parasite.

Automated analysers offer rapid, sensitive and cost effective screening of all samples. The automation in haematology helps in analysing various parameters which can help pathologist for a more diligent search for malarial parasites in peripheral blood films thus help immediate start of specific treatment to the patient.[1]

Automated hematology analyser named cell dyne ruby which was used in our study detect changes in volume and scatter properties of monocytes and lymphocytes which have been activated by malarial parasite.[2] Abnormal WBC histogram peaks at the threshold generating a "suspect malaria" flags are also evident. Malaria diagnosis with flow cytometry based hematology analyzers could become an important adjuvant diagnostic tool in routine laboratory work up of febrile patients in or returning from malaria endemic regions[3].

Expert light microscopy remains the "gold standard" for malaria diagnosis in most clinical settings.[1]

Materials and Methods

The study was conducted in the laboratory of Tertiary Care Hospital 12 months of duration from November 2014 to November 2015. 775 samples from the patients who presented to emergency department and for whom the attending clinicians had requested full blood count and malaria smears were included in the study.

Samples were processed as part of normal routine cell dyn ruby analyser for FBC (Full Blood Count). In our study, inadequate EDTA blood samples, clotted samples were excluded. In our study, we had used histograms and scatterplots which were generated by cell dyn ruby and interpreted the histograms in malarial cases.

Inclusion criteria: In present study, we accepted adequate quantity of samples. We included histograms above 2 purple colored dots (pink flag).

Exclusion criteria: In present study, we excluded clotted and insufficient blood samples. We also excluded histograms and scatterplots with one purple colored dots (pink flag).

Abnormal scattergram and abnormal WBC peak were observed diligently & compared with normal controls. (figure1)

Granlrty/Lobularity: The granularity (90°D scatter) information is plotted on the Y axis and the lobularity (90° scatter) information is plotted on the X axis.

Cell dyn ruby plots were interpreted as positive for malaria if 2 or more purple coded events – Pink flag (pigment containing monocytes, Figure 2) were detected in eosinophil area of lobularity / granularity plot as seen in color monitor of instrument. (Figure 2) The WBC subpopulations were further identified by the following colours: Neutrophils — yellow, Lymphocytes — blue, Monocytes — purple, Eosinophils — green, Basophils — white.

1. **Mononuclear polymorphonuclear separation:** The scatter information is plotted with the 90° scatter on the Y axis and the 10° scatter on the X axis. The mononuclear cells fall in the cluster in the lower left corner of the scatterplot and the polymorphonuclear cells fall in the cluster above and to the right of them.
2. **Neutrophil eosinophil separation:** The scatter information is plotted with 90°D scatter on Y axis & 90° scatter on X axis (granularity / Lobularity). Only polymorphonuclear cells are plotted on this scatterplot. The neutrophils fall in the lower of two clusters & the eosinophils fall in upper plot.
3. **NWBC-LYM-MONO Histogram:** The scatter information is plotted in a histogram format with the relative number of cells on the Y axis and the NWBC, Lymphocyte and Monocyte size distribution data on the X axis. In our study, atypical lymphocytes were present also found in positive malarial cases.
4. **Mono-Poly Histogram:** The scatter information is plotted in a histogram format with the relative

number of cells on the Y axis and the mononuclear and polymorphonuclear size distribution data on the X axis.

5. **RBC histogram:** The size distribution data for the red cells is displayed graphically as a histogram using 0° data. The size distribution data is on the X axis. The relative number of cells is normalized and plotted on the Y axis. In our study, RBC peak before 60 on x axis and decreased peak had been considered as anaemia. (figure4 for decreased RBC peak)
6. **Platelet histogram:** The scatter information is plotted in a histogram format the relative number of cells on Y axis and size distribution data on the axis. In our study, decreased platelet peak before 120 had been considered as thrombocytopenia. (Fig. 4 for platelet peak before 120 on x axis)

Peripheral Smear Examination: Peripheral smears were fixed with alcohol and then stained with routine Giemsa and some were stained with field stain also. As in urgent sample we used field stain. Thick & thin blood films were prepared and stained with Giemsa. All stained slides were examined by pathologist.

Result

Total 775 cases were studied, out of which 409 cases are positive for malaria which are confirmed by peripheral smear examination.

Out of 409 cases of malaria, Plasmodium vivax cases were 228 and Plasmodium falciparum cases were 169 cases. Mixed infection means patient having both P.Vivax and P.Falciparum. In our study 12 cases of mixed infection found. (Table 1)

Table 1: Total 409 cases of malaria

P.Falciparum		P.Vivax		Mixed infection	
Cases	Percentage	Cases	Percentage	Cases	Percentage
169	41.32%	228	55.74%	12	2.93%

In our study, majority of cases were found between 21-30 years of age for malarial infection. Maximum 60 cases were positive for P.Vivax malaria and 51 cases were positive for P.Falciparum malaria in the age group of 21-30 years. (Table 2)

Table 2: Distribution according to AGE for malaria

Age	Before 1 year	0-10 years	11-20 years	21-30 years	31-40 years	41-50 years	51-60 years	61-70 years	>70 years
P. Vivax	3	9	37	60	30	27	37	20	5
P.Falciparum	0	12	30	51	31	19	14	8	4
Mixed infection	0	0	1	3	4	4	0	0	0

In our study, Anaemia was more commonly seen in P.Falciparum malaria. Out of 169 cases, Anaemia was found in 97 cases (57.39%) of P.Falciparum. While, out of 228 cases, Anaemia was present in 68 cases (29.82%) of P.Vivax cases. In case of mixed infection, Anaemia was present in 6 cases (50%). (Table 3)

Table 3: Flag of cell count for malaria

Flag	P.Vivax (228 cases)		P.Falciparum (169 cases)		Mixed infection (12 cases)	
	Cases	%	Cases	%	Cases	%
Anaemia	68	29.82	97	57.39	6	50
Leukopenia	41	17.98	41	24.26	4	33.33
Thrombocytopenia	218	95.61	145	85.79	11	91.66
Monocytosis	142	62.38	111	65.68	7	58.33
Pink Flag	206	90.35	145	85.79	12	100

In our study, Leukopenia was present in 41 cases (17.98%) out of 228 cases of P.Vivax cases, 41 cases (24.26%) out of 169 cases of P.Falciparum and 4 cases (33.33%) out of 12 cases of mixed infection. (Table 3)

In our study, Thrombocytopenia was more common in P.Vivax malaria. It was seen in 218 cases (95.61%) out of 228 cases of P.Vivax, in 145 cases (85.79%)out of 169 cases of P.Vivax and in 11 cases (91.66%) out of 12 cases of mixed infection. (Table 3)

In our study, Monocytosis was more common in P.Falciparum. It was seen in 111 cases (65.68%) of P.Falciparum out of 169 cases, while in 142 cases (62.38%) of P.Vivax out of 228 cases and 7 cases (58.33%) out of 12 cases. (Table 3)

In our study, pink flag was regarded as more than 2 purple dots in WBC scattergram which was due to malaria pigment which was ingested by monocytes and neutrophils, and depolarizing the birefringent light .Then this refracted light was detected by cell dyn ruby (Table 3)

In our study, 206 cases (90.35%) out of 228 cases of P.Vivax malaria had pink flag and in case of P.Falciparum malaria, 145 cases (85.79%) out of 169 cases were having pink flag. In mixed infection all 12 cases (100%) had pink flag. (Table 3)

Table 4: Cell Shape in malaria

Flag	P.Vivax (228 cases)		P.Falciparum (169cases)		Mixed infection (12 cases)	
	Cases	%	Cases	%	Cases	%
Atypical Lymphocytes	134	58.77	129	76.33	11	91.66
Atypical Eosinophils	183	80.26	138	81.65	11	91.66

In this study, atypical lymphocytes were found most commonly in 129 cases (76.33%) out of 169 cases P. falciparum cases and in 11 cases (91.66%) out of mixed infection of malaria. In P.Vivax atypical lymphocytes was less common as compared to P.Falciparum malaria. (Table 4)

In our study, atypical eosinophils was present in majority cases of malaria. Incidence was seen in 183 cases (80.26%) out of 228 cases of P.Vivax, in 138 cases (81.65%) out of 169 cases of P.Falciparum and in 11 cases (91.66%) out of 12 cases positive for mixed infection. (Table 4)

In present study, abnormal WBC scattergram was present in almost all cases of 226 cases (100%) of P.Vivax and 12 cases (100%) of mixed infection of malaria and 163 cases (96.49%) out of 169 cases of P. falciparum malaria. (Table 5)

In present study, abnormal WBC peak is found in majority of malarial cases. It is present in 208 cases (91.22%) out of 228 cases of P.Vivax, 157 cases (92.89%) out of 169 cases of P. falciparum and in all 12 cases (100%) of mixed infection of malaria.(Table 5)

Table 5: Results for Abnormal WBC scattergram and Abnormal WBC peak for malaria

Flag	P.Vivax (228 cases)		P.Falciparum (169cases)		Mixed infection (12 cases)	
	Cases	%	Cases	%	Cases	%
Abnormal WBC scattergram	226	99.12	163	96.49	12	100
Abnormal WBC peak	208	91.22	157	92.89	12	100

We can find that histogram for platelet is an important predictor for diagnosis for malaria as sensitivity in P.Vivax 93.42% in P.Falciparum 86.39% and mixed infection 91.66%. (Table 6)

Table 6: Results for RBC and Platelet flag interpretation for malaria

Flag	P.Vivax (228 cases)		P.Falciparum (169cases)		Mixed infection (12 cases)	
	Cases	%	Cases	%	Cases	%
RBC peak before 60 on x axis	40	17.54	63	37.27	3	25
Decreased Platelet peak before 150 on x axis	213	93.42	146	86.39	11	91.66

In our study total cases are 775 cases.

True positive cases: 401 cases which are positive for malaria on histogram and also positive on peripheral smear examination.

False positive cases: 26 Cases which are positive for malaria in histogram but negative on peripheral smear examination.

True negative cases: 340 cases which are negative in both histogram and peripheral smear examination.

False negative cases: 8 cases which are negative in histogram but positive on peripheral smear examination.

Sensitivity = True positive / True positive + false negative x 100

Specificity = True negative / True negative + False positive x 100

Accuracy = true positive + true negative /true positive+ false positive + True negative+ false negative x 100

In our study,

Sensitivity = 401/401+8 x 100 = 98.04%

Specificity = 340/340+26 x 100 = 94.44%

Accuracy = 401+340/26+401+340+8 x 100 = 95.61%

Discussion

The worldwide accepted "gold standard" method for the diagnosis of malaria is the microscopic examination of peripheral smears[3]. This method is difficult, time-consuming, and, particularly at low level of parasite index which have to be examined by expert pathologist.[4]

Automation in hematology has helped in analyzing various parameters which may aid the pathologist for a search for the malarial in the peripheral smear, and guide for rapid diagnosis and also for prompt treatment. In such a way it is also useful for clinician[5,6].

In the majority of malarial infections, the Hb decreased and the anemia is related to the cumulative parasite density[7]. Possible causes of this anemia are increased hemolysis and a decreased rate of erythrocyte production[8]. Leukopenia was found to be a common finding in nonimmune patients with malaria[8]. Cell-Dyn ruby is used as autoanalyser for malaria detection by complete analysis of all blood samples. Detection of malaria pigment (hemozoin pigment) has been basis for diagnosis of malaria parasites[9,10].

The presence of purple dots (PCE) as Monocytosis above the separation line which called pink flag in present study and green dots as eosinophils is a highly specific sign of the presence of malaria in particular blood sample.[11,12]. Malarial pigment-containing monocytes may remain in the blood circulation for few weeks or months thus the abnormal histogram detected in cell dyn ruby which is used in present study can persist for some time despite clinical improvement & absence of parasite[11]. Thus observed abnormal histogram and WBC scattergram changes may not necessarily suggest acute infection of malarial disease as it is not specific for malaria.

In present study, abnormal WBC scattergrams and histograms seen in226 cases (99.12%) out of 228 cases of P. vivax and in 163 cases (96.49%) out of 169 cases of P. falciparum malaria and in all 12 cases of mixed infection of malaria(Table 7)

Aminder et al studied abnormal WBC scattergram in all 176 cases (100%) of P.Vivax and 24 cases (100%) of P.Falciparum cases. Sysmex 2100 et al study found 65 cases (15.7%) out of 413 cases of P.Vivax malaria as it had low sensitivity as compared to our study and other studies. In our study, 226 cases (99.2%) out of 228 cases of P.Vivax and 163 cases (96.49%) out of 169 cases of P.Falciparum were found positive for abnormal WBC scattergram. We found some variation due to presence of false negative cases in our study. (Table 7)

Table 7: Comparison of sensitivity of abnormal WBC scatter grams and histogram with other studies

Malaria diagnosis	Present study				Aminder et al study[2]				Sysmex 2100et al study[16]		
	P.Vivax (228 cases)		P.Falci (169 cases)		P.Vivax (176 cases)		P.Falci (24 cases)		P.Vivax (413 cases)		P.Falci
Ab. WBC scattergram	226	99.2%	163	96.49%	176	100%	24	100%	65	15.7%	-

In Aminder et al study, cases with presence of Atypical eosinophils in P. vivax 53.40% and in P.Falciparum 16.66% which is not comparable with present study in which 183 cases out of 228 cases. It seems from this observation, their exclusion criteria for sample selection is different from our study. (Table 8)

Table 8: Comparison of results of presence of Atypical eosinophil

Malaria diagnosis	In present study				In Aminder et al study[2]			
	P.Vivax (228 cases)		P.Falciparum (169 cases)		P.Vivax (176 cases)		P.Falciparum (24 cases)	
Atypical eosinophilia	183	80.26%	139	82.24%	96	53.40%	4	16.66%

Atypical lymphocytes flag is due to activated lymphoid cells present in cases of malaria.[13] Ho et al., hypothesized that, during P. falciparum malaria, the depression of cell-mediated immunity may favour the appearance of opportunistic viral infections which could be results of atypical lymphocytes. (Table 9)

In our study, flag for Atypical lymphocyte is present in majority cases of P.Falciparum malaria 129 cases (76.24%) out of 169 cases which is comparable with other studies. In Aminder et al study showed 16 cases (66.66%) out of 24 cases of P.Falciparum malaria. In Monika Jain et al study reveal 6 cases (75%) out of 8 cases of P.Falciparum malaria. (Table 9)

In present study, leucopenia is less common in both forms of malaria as compared to other studies as in present study we have taken more number of cases as compared to other studies. (Table 9)

Monocytosis in malaria indicates the increased activity of reticulo-endothelial system. Although monocytosis can be seen in other febrile infectious conditions it is particularly important to look for presence of pink flag which can help to suspect & detect malarial parasite[14]. (Table 9)

In present study, Monocytosis is seen with in P.Vivax 142 cases (62.28%) out of 228 cases and in P. falciparum 111 cases (65.38%) out of 169 cases which is comparable with Aminder et al study. However, it is not comparable with Monika Jain et al study because they have taken less number of cases and they studied histogram in cancer patients. (Table 9)

Table 9: Comparison of results of presence of Atypical lymphocytes, Leucopenia, Monocytosis, thrombocytopenia & Anaemia

Malaria diagnosis	Present study				Aminder et al study[2]				Monika jain et al study[9]			
	P.Vivax (228 cases)		P.Falci (169 cases)		P.Vivax (176 cases)		P.Falci (24 cases)		P.Vivax (31 cases)		P.Falci (8 cases)	
Atypical Lymphocyte	134	58.37%	129	76.24%	100	56.81%	16	66.66%	11	35%	6	75%
Leucopenia	41	17.98%	41	24.86%	86	48.36%	10	41.66%	13	42%	3	37.5%
Monocytosis	142	62.28%	111	65.38%	80	45.45%	10	41.66%	2	6%	1	12%
Anaemia	68	29.82%	97	57.39%	76	43.18%	12	50%	9	29.03%	4	50%
Thrombocytopenia	218	95.6%	145	85.79%	169	96.02%	15	62.5%	23	74%	4	50%

In present study, we found that anaemia is more common in P.Falciparum than in P.Vivax malaria. In our study anaemia is seen in 97 cases (57.39%) out of 169 cases of P. falciparum cases as compared to 68 cases (29.82%) out of 228 cases in P.Vivax malaria. Our results are comparable with other studies those are concluded the same results.

Thrombocytopenia is an important predictor to diagnosis of malaria. In our study it is found in 218 cases (95.6%) out of 228 cases of P.Vivax cases and in 145 cases (85.79%) out of 169 cases out of 228 cases of P.Falciparum. These results are comparable with other studies.

In our study 8 cases were negative on histogram but found positive on peripheral smear examination. In present study ≥ 2 PCE (purple coded events) rather than ≥ 1 PCE was used as cut off point. In these cases (8 false negative cases) a single PCE was noted above calculated line between the eosinophil and other WBC population. Due to their minimal difference in degree of granularity (Y axis = 90 degree depolarization light scatter) as

compared to normal monocyte population this was regarded as spurious events.[17]

In sample positive for malaria pigment, usually ≥ 2PCE were noted that showed a much higher degree granularity. When applying ≥ 1 PCE as criteria for positivity, all 8 false negative samples would have been regarded as positive.

In our study 26 cases were found false positive. It means that 26 cases are positive for malaria on histogram but found negative on peripheral smear examination. The main reason for false positive samples in our study results may be persistence of hemazoin containing monocytes after parasite clearance, pigment containing WBC were still present in more than 70% of patient after parasite clearance. Clearance time of pigment containing monocytes has been reported to be up to 2-3 weeks[15,16].

In present study, sensitivity is 98.04% and specificity is 94.44%. It is comparable with many studies. The differences in sensitivity and specificity of WBC scatterplots in detecting malaria cases in present study as compare with other studies may be due to many factors.

Unfortunately, the price for purchase and maintenance of this sophisticated instrument is beyond the means of many countries where malaria is endemic, and is thus a significant limitation.

Table 10: The higher sensitivity (98.04%) and specificity (94.44%) in our study compared with other studies

Studies	Sensitivity	Specificity
Present study	98.04%	94.44%
Thomas (Portugal) et al study[18]	72%	98%
Aminder singh et al study[2]	98%	94%
Sysmex 2100 et al study[16]	46.2%	99.7%
Briggs et al study[17]	98%	94%
Mandelow et al study[6]	72%	98%
Hanscheld et al [12]	95%	88%
Monika jain et al [9]	82%	100%

Conclusion

Our findings suggest that this novel method of Histogram study in malaria is a useful addition to conventional microscopy. Most importantly, it may permit the automated diagnosis of cases where no clinical request was made other than an FBC.

Although automated analysers are not a screening tool for evaluation of malaria; most of the laboratories in India have cell counters but awareness is lacking regarding specific patterns of malarial infection in the histogram & scatter gram. With the knowledge of these graphs & patterns paramedical staff, resident doctors, senior pathologists & even treating physicians might not miss malarial parasites even in the absence of a clinical request.

The great advantage of these plots are that haematopathologists can review the haematological data and the scatter plots on the analyser and see the slides again even if it is missed in initial screening.

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