Scatterplot Variations Seen in Malaria Using Automated Hematological Analyzers: A Series of Ten Cases

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Abstract

Background: Malaria is a major health problem in India. Complete blood count and peripheral blood smear (PBS) are important for its diagnosis. Interobserver variation makes PBS fallible. Rapid diagnostic tests cannot detect low parasitemia and mixed infections. Scatterplot from automated analyzers have shown variations previously which might be exploited. **Methods:** This descriptive study was conducted between July and August 2018. Scatterplot patterns of ten samples of confirmed malaria and 100 control samples were derived using automated hematology analyzers All other infections were ruled out by relevant culture and serology. Each malarial scatterplot was compared with the control pattern for abnormalities and their frequency was noted. **Results:** All ten samples belonged to the *Plasmodium vivax* species. Abnormalities detected included split in neutrophilic region, eosinophil-neutrophil merge, neutrophil graying, lymphopenia, ghost red blood cells eosinophil split, reactive lymphocytes, monocytosis, pseudoeosinophilia and neutrophilic leukocytosis. **Conclusion:** Variations in scatterplot patterns are seen in malaria and provide clues to the diagnosis of malaria.

Key Words: Hematologic Tests; Diagnosis; Malaria (Source: MeSH-NLM).

Introduction

Malaria is a major health care problem in India. In the World Malaria Report 2020 produced by the World Health Organization (WHO), India currently accounts for 3% of the global malaria burden and contributes to 86% of total malaria cases in Southeast Asia.[1] *Plasmodium falciparum* and *Plasmodium vivax* are the dominant species responsible for the spread, with both being reported in almost equal proportions in India and varying based on regions.

The primary investigations ordered in suspected malaria include a complete blood count and peripheral blood smear (PBS) besides other serological and microbiological analyses. While these investigations are admirable and help in identifying a large case load, the true burden of the disease is estimated to be much higher than the above number. PBS examination remains a tedious process which is time consuming and subjective, based on the expertise of the examining person.[2] Low detection levels, especially at low parasite levels, limits the accuracy of a microscope. Expertise may bring about variations, with the most experienced microscope users detecting numbers as low as 5 parasites/μL while the average user detects 50 parasites/μL.

Asymptomatic cases with low parasite numbers may thus be underestimated.[3] As much as 25% of malaria cases may be missed by microscopy.[4] Rapid diagnostic tests (RDT), on the other hand, are a poor choice in cases having low density parasitemia and mixed infection with twin malaria species.[5] Performance may also be affected by temperature and humidity variations which damage the nitrocellulose membrane and bound monoclonal antibodies of RDT, thus affecting its performance.[6]

Modern hematologic analyzers work largely on two principles: optical scatter which measures the deviation in the pathway of light caused by the size and granularity of the cell and electrical impedance which measures the change in electric current caused by blood cells.[7] In a study previously conducted by us, we have shown how acute febrile illnesses caused by an infectious etiology have shown variations in scatterplot patterns obtained from automated hematologic analyzers.[8] In particular, numerous studies have been conducted showing cell abnormalities represented in peculiar ways in the scatterplots of malarial patients, with species identification also possible.[9-11] In this study, we report on ten cases of malaria, confirmed on peripheral blood smear examination which showed unique scatterplot patterns. We aim to highlight these new features of scatterplot patterns associated with malaria infection.

Methods

This descriptive study was completed in the hematology laboratory of Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India between July and August 2018. K2 EDTA blood samples of cases with PBS and microbiologically confirmed malaria were taken as study samples and samples with no history of fever and normal white cell counts and differentials were taken as control. Since the study was performed on blood samples taken as part of a routine investigation and patients remained anonymous, ethics approval was waived by the Institute Ethics Committee. A total of ten cases of malaria diagnosed during the time period along with 100 normal samples were studied in the automated Sysmex XT2000i hematology analyzer. A simultaneous culture and serology were done for the control samples to rule out any hidden infection which may cause variation in scatterplot pattern.

In each case, 2 mL of EDTA venous blood was collected and analyzed by automated analyzer. In each case the complete blood counts and the scatterplot patterns were studied. Comparison of each scatterplot generated from these cases was done with the prototype control pattern (*Figure 1*) and the abnormalities were noted. The PBS was

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stained by Leishman stain with 2 minutes fixation and 15 minutes staining and studied in details for the morphology of the blood cells and presence of the malarial parasites.

Results

A total of ten cases of malaria and 100 controls were collected and their scatterplots generated using the Sysmex XT2000i analyzer. Out of the ten cases, five were taken from one of our earlier studies on scatterplot and acute febrile illnesses conducted around the same time.[8] The representative control normal scatterplot pattern used is shown in *Figure 1*.

All cases of malaria were of the *Plasmodium vivax* species and were confirmed by both positive RDT and the presence of trophozoites in the peripheral blood smear. The following findings were noted:

1. A split in the neutrophil region was evident in 5 of the 10 samples. This was represented by a change in shape of the light blue color from the normal ellipse to a double ellipse joined at the ends.
2. A merging of the eosinophilic region with the neutrophilic region was noted in 4 of the 10 samples. This was represented by the blue and the red population merging together without any space between them.
3. Graying of the neutrophil area was seen in 2 out of the 10 samples. While we are considering this as a separate entity, it may be considered a variant of the neutrophil-eosinophil merge with the only difference being an inability to recognize neutrophils and eosinophils as separate entities.
4. Lymphopenia was noted in 4 of the 10 samples. This was indicated by:
   - -Decrease in the area occupied by the pink color
   - -Decrease in the intensity of the pink color
5. Increased ghost red blood cells (RBC) were noted in 4 of the 10 samples. This was experienced by an increase in the area or intensity of dark blue color which was greater than two divisions on the x-axis.
6. A split in the eosinophil population was noted in 3 of the 10 samples. This presented in the scatterplot as two populations of red color separated by a band of black color either in the x-axis or y-axis.
7. Reactive lymphocyte populations were seen in 2 samples by a shower of pink cells which were present over the green monocytic region.
8. Besides pseudoeosinophilia, both monocytosis and neutrophilic leukocytosis were each seen in 2 of the 10 samples.

A composite image highlighting all the findings has been shown in *Figure 2*.

Discussion

According to a WHO report in 2019, there were an estimated 409 000 deaths from malaria globally.[1] Hence, the diagnosis of malaria should be prompt and accurate so that treatment can be started in a timely manner to avoid unnecessary complications. PBS examination is often the first line of investigation in suspected cases of malaria and changes in the scatterplot pattern, if carefully identified, it can help in identifying the parasites earlier in the blood.

The key abnormalities found in our scatterplot analysis included neutrophil splitting, eosinophil-neutrophil merge, graying of neutrophil region, lymphopenia, ghost RBC increase and eosinophil split. Automated hematological analyzers are based on flow cytometry. Special fluorescent dyes are used to stain nucleic acids. The channel lyses RBCs along with platelets and binds the nucleic acid using a dye to give a fluorescence proportionate to the nucleic acid content. The higher the percentage of nucleic acids, the greater the intensity of scatterplot pattern.[12]

Figure 1. Representative scatterplot showing the pattern of white blood cells in a peripheral blood smear. The pink plot represents lymphocytes, green represents monocytes, light blue represents neutrophils, red represents eosinophils, and red represents red blood cells.
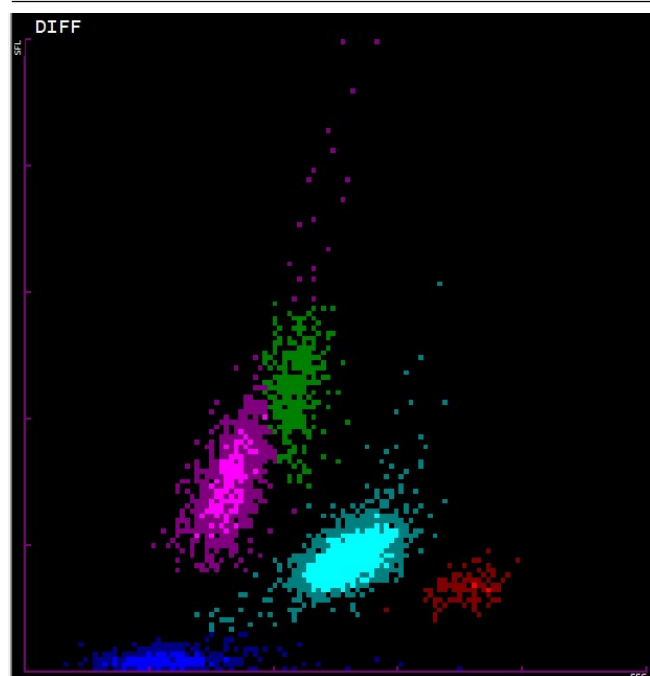
Figure 2. Scatterplot variations and their interpretations:1. Neutrophil and eosinophil merge, 2. Graying of neutrophils, 3. Lymphopenia, 4. Increase in RBC granularity, 5. Splitting of eosinophils, 6. Splitting of neutrophils, 7. Reactive lymphocytosis, 8. Monocytosis, 9. Leucocytosis.
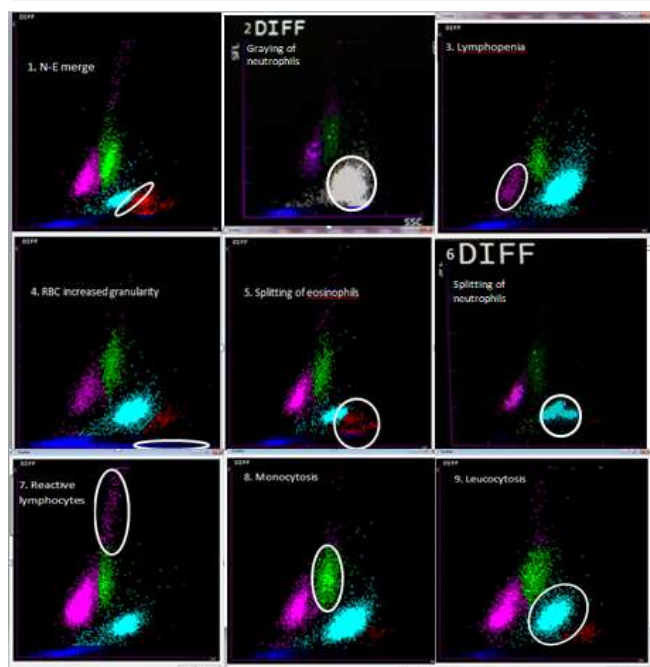
In all ten cases with scatterplot abnormalities, schizonts of *Plasmodium vivax* was seen in the peripheral smears, which was expected considering the low incidence of *Plasmodium falciparum* in Pondicherry.[13] Specific changes have been observed in *Plasmodium vivax* because of the presence of hemozoin pigments of the schizonts in the peripheral blood. These changes have been more often seen in

Plasmodium vivax than in *Plasmodium falciparum*, as schizonts are usually not seen in the peripheral blood in the latter.[14,15] This may be a limitation of this detection method, as *Plasmodium falciparum* infections are missed by scatterplot investigations.

Earlier studies have found changes such as pseudoeosinophila and the graying of neutrophilic areas as relevant findings, which make it pertinent to check for malarial parasites in peripheral blood smear.[16,17] We were evidently able to concur such findings in our study. Apart from that, we were able to obtain unique findings of increased ghost RBC density, lymphopenia, and a dual population of eosinophils represented by a split in the eosinophilic region, which we found to be a good pointer of malaria. In 50% of our cases, neutrophil population split was found. This could be a potentially strong indicator of malaria. Neutrophilic merge with the eosinophilic region was also a unique finding, as was eosinophil split. Reactive lymphocytosis and monocytosis are findings in a number of other illnesses but should also arouse a strong suspicion of malaria if it can be correlated clinically and epidemiologically as malaria.

A study conducted by Huh et al in South Korea extensively studied 144 cases of Plasmodium vivax malaria and found a high incidence of pseudoeosinophilia characterized by a difference in the eosinophil count detected by the analyzer and observed on the smear.[18] This was found to be as high as 39%. In our study, we only detected two samples with pseudoeosinophilia, showing that pseudoeosinophilia may not be a very sensitive finding. This is in line with other Indian studies which have found spurious eosinophilia in 1.5-4% of the population.[9,19] The same study by Huh et al also found similar findings of dual eosinophil

and neutrophil population; however, their incidence was very low compared with the pseudoeosinophil population. In total, they found 52.10% scattergrams being abnormal, indicating their importance in diagnosis.[18] A follow up study conducted by Yoo et al in 2010 found abnormalities in 15.70% of the scattergrams, despite finding the same incidence of pseudoeosinophilia.[20] In fact, these authors hypothesized that pseudoeosinophilia, neutrophil clusters and neutrophil-eosinophil merge were largely resulting from the hemozoin pigments in neutrophils and shouldn't be considered a separate entity. While studies by Huh et al. and Yoo et al. have found 52.10% and 15% of abnormal, in our study we found all ten of our samples to report some kind of abnormal scatterplot, which was in line with the Indian studies mentioned earlier that found 100% and 83.8% abnormal scatterplot patterns.[9,19] Thus, being aware of these scatterplot findings in the presence of clinical suspicion may help in the early diagnosis and initiation of treatment in malaria. A limitation of our study is the small sample size, yet it constitutes a sizeable number compared to the annual incidence of malaria in Puducherry.

Conclusion

Scatterplot patterns in malaria have been reported with varying sensitivity and specificity. We found abnormal scatterplot patterns in all the 10 cases of *Plasmodium vivax* malaria some of which have not been described before. These patterns should be kept in mind by the pathologist or the laboratory personnel and should prompt a thorough screening of the peripheral blood film to confirm for the parasites. When supplemented with peripheral blood smear examination, they are an adjunct to the current diagnostic modalities.

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