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Original Article

Pathology Section

Malaria Diagnosis Using Automated Analysers: A Boon for Hematopathologists in Endemic Areas

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ABSTRACT

Background: Haematological abnormalities are common in acute febrile tropical illnesses. Malaria is a major health problem in tropics. In endemic areas especially in the post monsoon season, it is not practical to manually screen all peripheral blood films (PBF) for malarial parasite. Automated analysers offer rapid, sensitive and cost effective screening of all samples.

Aim: The study was done to evaluate the usefulness of automated cell counters analysing their histograms, scatter-grams and the flaggings generated in malaria positive and negative cases. The comparison of other haematological parameters were also studied which could help to identify malaria parasite in peripheral blood smear.

Materials and Methods: The blood samples were analysed using Beckman coulter LH-750. The abnormal scatter grams and additional peaks in WBC histograms were observed diligently & compared with normal controls. Haematological abnormalities were also evaluated.

Statistical Analysis: Statistical analysis was done by using software Epi-Info version 7.1.4 freely available from CDC website. Fisher exact test was applied to calculate the p-value and value < 0.05 was considered as significant. Final identification of malarial parasite species was done independently by peripheral blood smear examination by two pathologists.

Results: Of all the 200 cases evaluated abnormal scatter grams were observed in all the cases of malaria while abnormal WBC histogram peaks were noted in 96% cases demonstrating a peak at the threshold of the histogram. The difference between number of slides positive for abnormal WBC scatter gram and abnormal WBC histogram peaks were statistically highly significant (p=0.007). So abnormal WBC scatter gram can better give idea of malarial parasite presence. Of the haematological parameters thrombocytopenia (92% cases) emerged as the strongest predictor of malaria.

Conclusion: It is recommended for haematopathologists to review the haematological data and the scatter plots on the analyser along with peripheral blood smear examination.

Keywords: Automation, Histogram, Malarial parasite, Scatter gram

INTRODUCTION

Haematological abnormalities are common in acute febrile tropical illnesses. Malaria is a major health problem in tropics. In endemic areas especially in the post monsoon season, it is not practical to manually screen all peripheral blood films (PBF) for malarial parasite. The automation in haematology helps in analysing various parameters which can help the pathologist for a more diligent search for malarial parasites in peripheral blood smear thus allow immediate start of specific therapy to the patient [1]. Automated analysers offer rapid, sensitive and cost effective screening of all samples. VCS technology of automated analysers detect changes in volume, conductivity and scatter properties of monocytes & lymphocytes which have been activated by malarial parasites & reflected as the heterogeneity of the volume in scatter grams along with infected RBC's depicted in the non WBC's areas of the scatter gram called ghost areas. Abnormal WBC histogram peaks at the threshold generating a "suspect malaria" flags are also evident. A flag to indicate the potential presence of malaria parasites could be a valuable diagnostic method for the detection of malaria and may become a routine parameter in the diagnosis [2]. Hence it was thought pertinent to analyze the coulter data with the peripheral smears in suspected cases of malaria.

MATERIALS AND METHODS

A total of 200 patients presented with fever or clinically suspected malaria, in Department of Clinical Pathology, Dayanand Medical College and Hospital Ludhiana during a period of 21 months (from March 2013 to November 2014) were included in the study.

Inclusion criteria: EDTA blood samples from both outpatient & admitted febrile patients with or without clinical suspicion of malaria were included in the study.

Exclusion criteria: Patients without history of fever, inadequate EDTA blood samples, clotted samples, peripheral blood smear showing normoblastemia. The informed consent was taken from patients by respective treating physicians.

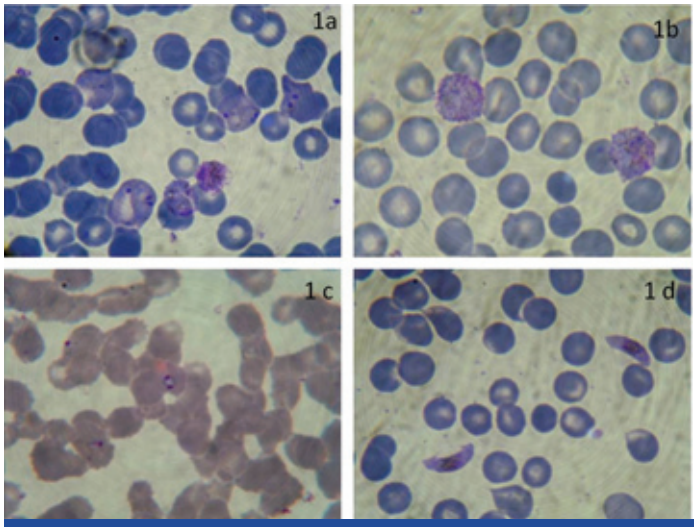
Institutional ethical standards have been followed however the ethical clearance was not required as the study includes the examination of blood samples given by patients for their routine check up without disclosing their identities. The blood samples were analysed using Beckman coulter LH-750. The abnormal scatter grams and additional peaks in WBC histograms were observed diligently & compared with normal controls. Haematological abnormalities like anaemia, leucopenia, thrombocytopenia, lymphocytosis, monocytosis, pseudoeosinophilia and reticulocyte count were also evaluated. Peripheral smears were fixed with 95% alcohol, and stained with routine Giemsa stain and were examined by at least two independent pathologists.

STATISTICAL ANALYSIS

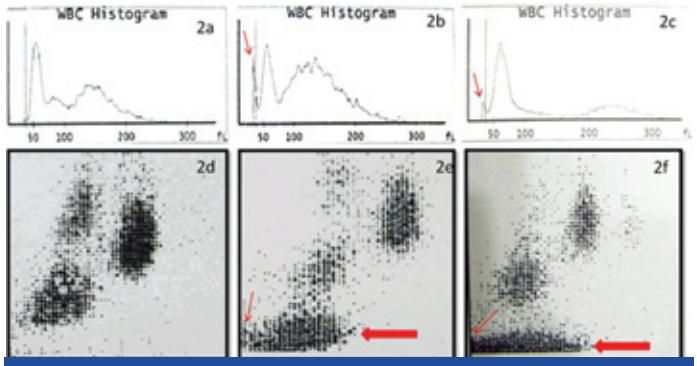
Statistical analysis was done by using software Epi-Info version 7.1.4 freely available from CDC website. Fisher exact test was applied to calculate the p-value and value < 0.05 was considered as significant. Final identification of plasmodium species was done independently by peripheral blood smear examination by two pathologists.

RESULTS

Of all the 200 cases evaluated, majority were males with male female ratio of 3.1:1. The mean age was 33.6 years with youngest being one-year-old female and eldest was 85-year-old male. Majority of the patients had *Plasmodium vivax* (88%) while *Plasmodium falciparum* was seen in 12% cases [Table/Fig-1a-d]. Abnormal scatter grams were observed in all the cases of malaria. Abnormal WBC



[Table/Fig-1a-d]: (a) Early Trophozoites of *P. vivax*.
(b) Schizonts of *P. vivax*.
(c) Trophozoites of *P. falciparum*.
(d) Gametocytes of *P. falciparum* (Giemsa X 400).



[Table/Fig-2a-f]: (a) Normal WBC histogram in malaria negative cases.
(d) Normal scattergram in malaria negative cases.
(b) Abnormal large straight peak before the threshold in *P. vivax* (Small arrow).
(e) Abnormal large ovoid scattergram in *P. vivax* at the bottom (Large arrow); Small arrow showing Partial blending with threshold line.
(c) Abnormal small peak before the threshold in *P. falciparum* (Small arrow).
(f) Abnormal large linear scattergram in *P. falciparum* at the bottom (Large arrow), Small arrow showing complete blending with threshold line.

histogram peaks were noted in 96% cases demonstrating a peak at the threshold of the histogram [Table/Fig-2a-f]. The differences have been observed in the patterns of these histograms & scatter grams in *P. vivax* & *P. falciparum* cases as shown in the figures. Thus these graphical patterns can also help in differentiating the malarial species. The X axis gave the cell size (fl) and y-axis the cell count, added to the advantage of automation in suspecting cases of malaria. The difference between number of slides positive for abnormal WBC scattergram and abnormal WBC histogram peaks were statistically highly significant. (p=0.007). So, abnormal WBC scatter gram can better give idea of malarial parasite presence.

Of the haematological abnormalities studied, thrombocytopenia (92% cases) emerged as the strongest predictor of malaria. The difference between number of slides positive for leucopenia, anaemia, eosinophilia, monocytosis were highly significant by comparing them with thromocytopenia (p<0.00001). Simultaneous reticulocyte stain was performed in all cases and reticulocyte count was done. Reticulocytopenia was noted in 99% of the cases which might be attributed to direct bone marrow depression due to malarial parasites. Reticulocytopenia was also statistically significant positive than thrombocytopenia (p<0.001). Hence, both Thrombocytopenia and Reticulocytopenia is better predictor for Malaria. Leucopenia was seen in 48% cases & anemia in 44% cases. Total leucocyte count ranged from 1.2 x 10^9/l to 11.5 x 10^9/l. Leucopenia was more common in present study. Lymphocytosis, monocytosis & immature granulocytes were also seen in many cases [Table/Fig-3]. The difference between number of slides

Flags	Total number of cases (n=200)	*P. Vivax* (n=176)	*P. Falciparum* (n=24)	Fisher Exact Test Chi-Value (p-value)
Cell Count				
Thrombocytopenia	184	169	15	Base
Leucopenia	96	86	10	90.01 (Very Highly Significant)
Anaemia	88	76	12	103.68 (Very Highly Significant)
Eosinophilia	3	2	1	325.37 (Very Highly Significant)
Monocytosis	90	80	10	100.2 (Very Highly Significant)
Reticulocytopenia	198	175	23	9.8 (p=0.001, Highly Significant)
Cell Shape				
Immature Granulocytes	100	96	4	0.81 (p=0.36, Non Significant)
Atypical Lymphocytes	110	100	10	
WBC Graph				
Abnormal WBC Scattergram	200	176	24	6.25 (p=0.007, Significant)
Abnormal WBC histogram peaks	192	176	16	

[Table/Fig-3]: Different abnormal haematological parameters in 200 cases

positive for immature granulocytes and atypical lymphocytes were not significant (p>0.05).

The pseudoeosinophilia was seen in only 3 cases. This was attributed to the high sensitivity of Beckman Coulter LH 750 as compared to other automated analysers. The average parasitemia (parasitic index) was ranging between 0.1% to 4.5%.

DISCUSSION

Malaria is a major health problem in the tropics. In endemic areas especially in post monsoon season, microscopic diagnosis, the established method for laboratory confirmation of malaria requires technical expertise and repeated smear examination. In the recent years, efforts have been made to replace the traditional blood film for the diagnosis of malaria. Polymerase chain reaction was found to be sensitive in the diagnosis of all 4 species of malaria. But the limitation is that, it is expensive and impractical for routine diagnosis of malaria [3,4].

The quantitative buffy coat method (QBC), a blood parasite detection method has been used in few laboratories as a backup to peripheral blood films. It has also been used as an initial screening technique also as thick and thin films are examined only on QBC-positive samples. This fluorescent method has few disadvantages because of high cost of the equipment and consumables & non-specificity of the fluorescent stain. The principal behind this method is; Howell-Jolly bodies will fluoresce with acridine orange stain but problem is that non–Plasmodium falciparum malaria detection rate is low because of denser late stages of parasites, thus they remain hidden in the mononuclear layer [4,5]. Automation in haematology has helped in analysing various parameters which may aid the pathologist for a more diligent search for the malarial parasite in the peripheral smear, and allow prompt institution of specific therapy [6,7].

Many authors documented that the Cell-Dyn 3500 was the first autoanalyser used for malaria detection by complete analysis of all blood cell lines. It was based upon principle that the WBC differential is generated when scattered laser light of white blood cells hit them

at four different angles [8-11]. Malaria pigment which was ingested by monocytes and neutrophils depolarizing the birefringent light which was detected by the instrument. The appearance of purple dots as monocytes above the separation line and green dots as eosinophils is a highly specific sign of the presence of ingested malaria pigment [12,13]. Pigment-containing monocytes may remain in the blood circulation for few weeks thus the changes detected in cell counters can persist for some time despite clinical improvement & disappearance of parasite [13]. Thus these observed changes may not necessarily indicate acute phase of malarial disease.

Use of the Standard deviation volume of monocytes and lymphocytes to flag for the possible presence of parasites of malaria has been suggested by Briggs et al., & Jain et al., [5,14]. In infected patients reactive changes occurred in lymphocytes and monocytes as increased cell size and therefore have increased volumes and SD of the volumes. The authors calculated and developed an algorithm called malaria factor by combing these changes. They used a cut-off value for the Malaria Factor of greater than 3.7, the specificity of which was 94% and sensitivity was 98%. This malaria factor was an indicator of malarial infection [5] but in the present study we focused only on the scattergrams and histograms along with haematological abnormalities only as malaria factor calculation was found to be cumbersome by our laboratory staff whereas the parameters evaluated in the study are easily interpretable and reproducible by medical students and technical assistants. In present study, abnormal WBC scattergrams and histograms seen in all the cases of *P. vivax* and more than half of *P. falciparum*.

Park et al., described 3 cases of pseudoeosinophilia by sysmex cell counter [15]. Pseudoeosinophilia in 6 of 16 malaria-infected patients (38%) was observed by Huh et al., [16]. Authors documented that WBC-DIFF channel abnormalities and pseudoeosinophilia were the result of haemozoin containing neutrophils. These cells because of their high side scatter properties were either placed in the eosinophil region giving rise to pseudoeosinophilia or they manifest as multiple neutrophil clusters because of fusion of neutrophil and eosinophil cluster regions. Thus the presence of schizonts and late trophozoites correlates well with the abnormal findings on WBC-DIFF scatter plot abnormalities. Because the pigment ingested by neutrophils is wrongly classified as eosinophils or as multiple neutrophil clusters. Red blood cells and the reticulocytes infected with malarial parasite have significantly increased nucleic acid content leading on to their prominent appearance in the RBC ghost area. No such significant findings were noted in the present study and this was credited to the high sensitivity of the Beckman Coulter LH 750 [15-17].

Reticulocytopenia in malaria infection is due to direct invasion, anaemia of chronic disorder and dyserythropoiesis. Erythroid precursor cells represent a common target for *Plasmodium falciparum*. *P. falciparum* can invade a large percentage of the RBC, whereas *P. vivax* is limited to reticulocytes. *P. vivax* invades only Duffy blood group-positive RBC and is largely limited to reticulocytes. The anaemia is typically normocytic and normochromic, with a notable absence of reticulocytes [18-21].

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 other cause of bone marrow depression leading to anaemia and reticulocytopenia [22]. We had seen reticulocytopenia in 99% of the cases of both species. Jain et al., studied the automation research work and found about the growing interest in the use of routine haematological blood analysis for presumptive diagnosis of malarial infection [14]. They documented that about the comparison between early and late forms of malarial parasites in relation to WBC-DIFF scatter plot abnormalities. They found significant correlation of late trophozoites with the WBC-DIFF scatter plot abnormalities, in comparison to early ring forms of malaria. In our study the difference between number of slides positive for immature granulocytes and atypical lymphocytes were not significant statistically while monocytosis was statistically significant. A study done by Akhtar et al., who studied in detail the haematological profile in malaria; clearly demonstrated that monocytosis had more statistically significant value as compared to lymphocytosis in both types of malarial infection as seen in our study too [23]. Monocytosis in malaria reflects the increased activity of reticulo-endothelial system. Although monocytosis can be seen in other febrile infections it is particularly important to look for presence of yellow brown pigmented material in monocytes which can help to suspect & detect malarial parasite. Leucopenia primarily reflects the state of hypersplenism according to Akhtar et al., and we found more number of cases of leucopenia as compared to leucocytosis. Increased WBC count particularly neutrophilia is due to secondary bacterial infection.

LIMITATIONS

There are few limitations of this study which includes non existence of any mixed malarial infection during specified time period of study to analyse graphical changes in them. Parasitic index was calculated but its correlation with other haematological parameters has not been assessed as it was not the part of aim of study.

CONCLUSION

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. However, these patterns when present could only suggest a diagnosis of malaria and should always be confirmed by a peripheral blood smear examination. 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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