

MALARIA: THE VALUE OF THE AUTOMATED DEPolarIZATION ANALYSIS

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Abstract. This retrospective and descriptive study was carried out in the University of Malaya Medical Center (UMMC) from January to September, 2004. This study aimed to evaluate the diagnostic utility of the Cell-Dyn 4000 hematology analyzer's depolarization analysis and to determine the sensitivity and specificity of this technique in the context of malaria diagnosis. A total of 889 cases presenting with pyrexia of unknown origin or clinically suspected of malaria were examined. Sixteen of these blood samples were found to be positive; 12 for *P. vivax*, 3 for *P. malariae*, and 1 for *P. falciparum* by peripheral blood smear as the standard technique for parasite detection and species identification. Demographic characteristics showed that the majority of patients were in the age range of 20-57 with a mean of 35.9 (\pm SD) 11.4 years, and male foreign workers. Of these, 16 positive blood samples were also processed by Cell-Dyne 4000 analyzer in the normal complete blood count (CBC) operational mode. Malaria parasites produce hemozoin, which depolarizes light and this allows the automated detection of malaria during routine complete blood count analysis with the Abbot Cell-Dyn CD4000 instrument. The white blood cell (WBC) differential plots of all malaria positive samples showed abnormal depolarization events in the NEU-EOS and EOS I plots. This was not seen in the negative samples. In 12 patients with *P. vivax* infection, a cluster pattern in the Neu-EOS and EOS I plots was observed, and appeared color-coded green or black. In 3 patients with *P. malariae* infection, few random depolarization events in the NEU-EOS and EOS I plots were seen, and appeared color-coded green, black or blue. While in the patient with *P. falciparum* infection, the sample was color-coded green with a few random purple depolarizing events in the NEU-EOS and EOS I plots. This study confirms that automated depolarization analysis is a highly sensitive and specific method to diagnose whether or not a patient has malaria. This automated approach may prove to be particularly useful in situations where there is little or no clinical suspicion of malaria.

INTRODUCTION

Malaria is the most important parasitic disease worldwide. Early diagnosis relies crucially on clinical suspicion. A clinician suspecting the disease has to explicitly request malaria smears. Lack of clinical suspicion is a well-known factor for a missed diagnosis, which contributes substantially to patient morbidity and mortality in this disease. A study from Canada showed that 59% of malaria cases were initially misdiagnosed and 16% required three or more physicians' contacts before malaria smears were ordered (Kain *et al*, 1998). Compared to hemoglobin, the malaria pigment, hemozoin, (produced by plasmodium parasites during late intraerythrocytic stages of infection), depolarizes light and significant amounts of hemozoin can be internalized by circulating neutrophils and monocytes. (Metzger *et al*, 1995; Arese and Schwarzer, 1997). Earlier reports have described how this intracellular malaria pigment in white blood cells (WBC) could be

detected during routine complete blood count (CBC) analysis by automated depolarizing measurement that is part of the WBC differential analysis performed by some Cell Dyn hematology instruments. (Mendelow and Coetzer, 1999; Fawzi *et al*, 2003). Further, recent reports with CD4000, have suggested that *P. vivax* infection may show some distinctive differences in depolarization patterns compared to *P. falciparum* (Fawzi *et al*, 2003; Scott *et al*, 2003). This study was therefore undertaken to determine the value of automated depolarization light patterns generated during white blood cell (WBC) differential analysis by the Cell Dyn 4000 instrument as an alternative technique for malaria detection (in terms of sensitivity and specificity) as compared to the existing conventional peripheral blood smear examination. In addition, this study was to evaluate differences in the depolarization patterns as seen in the different *Plasmodium* species. We believe that this is the first report from Malaysia where although malaria is endemic it is not frequently seen in an urban hospital like ours. Therefore, this novel approach may have the potential to alert medical technologists examining the scatterplots from a Cell Dyn 4000 instrument to suspect the possibility of malaria and initiate the examination of a peripheral blood smear for parasites, even in the absence of clinical suspicion.

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MATERIALS AND METHODS

A total of 889 cases presenting with pyrexia of unknown origin or clinically suspected of malaria were examined by peripheral blood smear microscopy for parasitic detection and species identification. All cases were also analysed with the Abbott Cell-Dyn 4000 instrument in the normal CBC (complete blood count) operational mode. The Cell Dyn 4000 is an automated flow cytometry based hematology analyzer that uses DNS fluorescence to evaluate white blood cell viability and 90° depolarization light analysis to identify and enumerate eosinophils. For this study the analyser configuration was standard and unmodified from that recommended by the manufacturers, and stability and optimal settings were checked with controls and calibrators as required. After sample processing, screen displays and color printouts of the Cell Dyn NEU-EOS (90° depolarized vs 90° polarized light scatter) and EOS I (90° depolarized vs 0° light scatter) leukocyte differential plots were examined for abnormal depolarizing patterns. In these plots, specific color coding of events is applied to neutrophil (orange), lymphocyte (blue), monocyte (purple), and basophil (black) populations. In addition to the occasionally observed minor basophil component, a black coding is also applied to particles that are considered nonleukocyte in type if their optimal characteristics do not accord with predefined criteria.

Statistical analysis

All the findings obtained were entered, edited and analyzed using statistical software SPSS version 10 (SPSS Inc, Chicago, Ill, USA). The data with quantitative variables were expressed as median and range, whereas, qualitative variables were expressed as frequency and percentage.

RESULTS

Of all the 889 cases presenting with pyrexia of unknown origin or clinically suspected of malaria, 16 samples were found to be positive for malaria: 12 for *P. vivax*, 3 for *P. malariae*, and 1 for *P. falciparum* by peripheral blood smear examination. The demographic characteristics of the 16 positive cases showed that the majority of them were in the age range of 20-57 with a mean of 35.9 (± SD) 11.4 years, and were male foreign workers (data not shown). The screen displays and color printouts of the white blood cell (WBC) differential plots of all 16 positive peripheral blood smears showed abnormal depolarization events in the NEU-EOS, and EOS I plots, which was not seen in the malaria negative samples (Fig 1).

The 12 patients with vivax malaria showed abnormal depolarizing green or black color-coded events with absence of depolarizing purple events in the NEU-EOS plot and a distinct cluster with a low size signature in the EOS I plot (Fig 2). The 3 patients with *P. malariae* infection showed random depolarization events in the NEU-EOS and EOS I plots which appeared color-coded green, black or blue (Fig 3). The single patient with *P. falciparum* infection showed few random purple and green events in the NEU-EOS and EOS I plots as shown in Fig 4. In addition, sensitivity and specificity of this Depolarization Laser Light technique were found to be 100% when compared to the conventional peripheral blood smear.

DISCUSSION

More than 10 years ago malaria pigment (hemozoin) was found to be birefringent, and it was predicted that this property could be used to automate

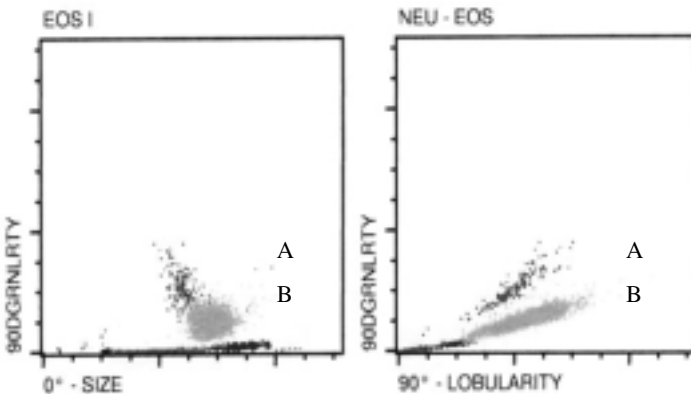


Fig 1- Normal pattern of malaria negative in CD 4000 NEU-EOS and EOS I plots illustrating the typical pattern of depolarization by eosinophils (indicated as A) and their separation from neutrophils (indicated as B).

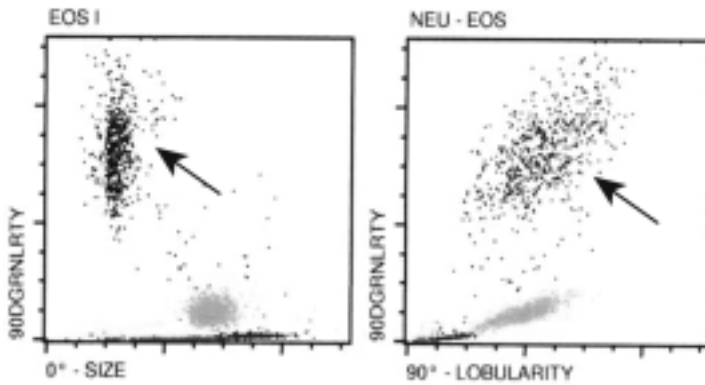


Fig 2- Positive pattern for *P. vivax* infection in CD 4000 EOS I plot illustrating distinct depolarizing black cluster with a low size signature and NEU-EOS plot illustrating abnormal depolarizing black color-coded events with absence of depolarizing (indicated by arrows).

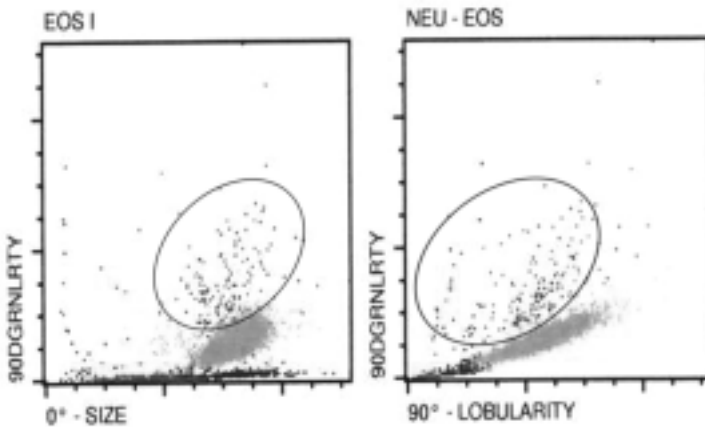


Fig 3- Positive pattern of *P. malariae* infection in CD 4000 NEU-EOS and EOS I plots illustrating random depolarization (indicated by circles).

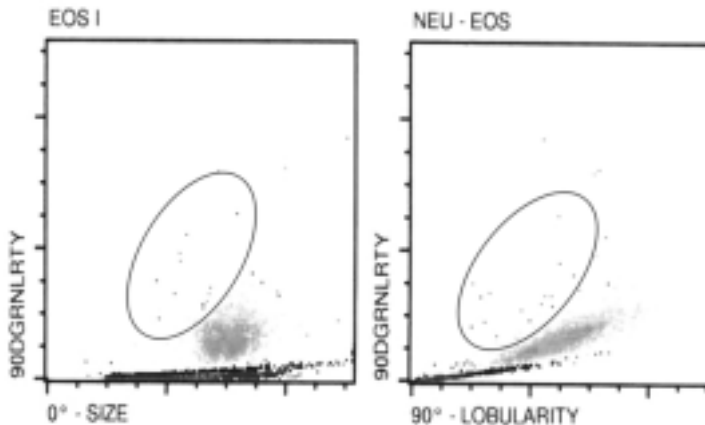


Fig 4- Positive pattern of *P. falciparum* infection in CD 4000 NEU-EOS and EOS I plots illustrating few random (indicated by circles).

the detection of malaria (Lawrence and Olsen, 1986). Malaria pigment is produced by the malaria parasites during intraerythrocytic development as the end product of hemoglobin digestion. When the malaria-infected red cells rupture, the parasites and hemozoin aggregates are released into the plasma, which is then ingested by circulating phagocytes (Metzger *et al*,

1995; Arese and Schwarzer, 1997). A South African study was first to document the automated detection of leukocyte hemozoin (Scoff *et al*, 2002). Subsequently, this approach has been extensively validated and is regarded as particularly valuable when complete blood count investigations are undertaken for febrile patients with no clinical suspicion of malaria

(Haenscheid *et al*, 1999, 2001; Coetzer *et al*, 2000). A previous study, however, indicated that convalescent malaria patients with no residual parasitemia also showed abnormal depolarization patterns due to the delayed clearance of pigment-containing leukocytes and therefore detection of intracellular hemozoin by Cell Dyn instruments should be interpreted as either active or recent malaria infection (Day *et al*, 1996). In addition, another study showed that there is no correlation between the percentage of parasitemia and the number of abnormal depolarization events; hence this approach cannot be used to monitor therapy (Mendelow and Coetzer, 1999).

In our study, although the number of positive malaria cases was small, they showed 100% sensitivity and specificity, as demonstrated in other studies (Coetzer *et al*, 2000; Scott *et al*, 2003). The examination of the Cell Dyn 4000 NEU-EOS and EOS I plots in our and other studies (Scott *et al*, 2003) revealed some variations in the depolarizing patterns (Scott *et al*, 2003). Seventy-five percent (12/16) of the cases in our study were *P. vivax* infections which showed the presence of atypical depolarizing green or black color coded events in the absence of depolarizing purple events in the NEU-EOS plot. These same samples formed a distinct clustered population of depolarization events of small size signature, relatively homogenous and located with optical and fluorescent characteristics consistent with permeabilized red blood cells in the EOS I plot. This was supported by one study, which suggested that these small sized depolarization events may selectively represent the proportion of late-stage schizonts/gametocytes in red cells rather than overall parasitemia per se (Fawzi *et al*, 2003).

The *P. falciparum* sample showed a different depolarizing pattern consisting of few random purple and green events in the NEU-EOS and EOS I plots. A recent study showed that the predominant pattern in *P. falciparum* infection cases was purple depolarizing events with or without non-purple (usually green or black) depolarizing particles. The signature size of this was similar to that of neutrophils and monocytes and thus corresponded to neutrophils and monocytes with ingested malaria pigment (hemozoin) (Fawzi *et al*, 2003). This is supported by another study which revealed that with *P. falciparum* infection, much of the pigment enters the plasma compartment on rupture of the red cell schizont although some remains associated with the macrogametocyte (Jamjoom, 1988). Intracellular hemozoin in neutrophils has been suggested as a measure of disease severity in malaria, as the proportion of pigment-laden neutrophils is higher

in cases of cerebral malaria compared to patients with mild infections (Nguyen *et al*, 1995; Amodu *et al*, 1998). In contrast, the proportion of pigment-containing monocytes appears to have a lower degree of clinical association (Amodu *et al*, 1997). It has been suggested that the ingested hemozoin may be responsible for the impaired immune responses and reduced effectiveness of antigen presentation that is often characteristic of *P. falciparum* infection (Scott *et al*, 2003). In addition, other studies have shown that malaria infected red cells in *P. falciparum* do not contain sufficient depolarizing material to be detected by automated depolarization analysis and the same appears also to be true for *P. falciparum* gametocytes (Mendelow and Coetzer, 1999; Scott *et al*, 2003). Over the years, there has been increasing interest in the process of pigment formation by *P. falciparum* as a possible target for antimalarial drugs (Warhurst, 1995). Chloroquine and other quinoline compounds inhibit hemozoin production in chloroquine-susceptible *P. falciparum*, but not in chloroquine-resistant *P. falciparum* malaria (Egan *et al*, 1994). These drugs appear to act by inhibiting heme polymerase activity (Slater and Cerami, 1992), preventing the formation of hemozoin and thus exposing the parasite to toxic heme.

In the case of *P. malariae* infection, we suggest that more studies need to be carried out to identify its abnormal depolarization patterns and their implication in this species, as it appears that currently there is no study which can provide this information.

In conclusion, this study confirms that automated depolarization analysis implemented as part of the routine WBC differential analysis by the Cell Dyn 4000 provides an alternative method for malaria detection. The study also confirms previous reports that the abnormal depolarization patterns are different in *P. vivax* and *P. falciparum* which is probably due to the different cellular location of malarial pigment in blood cells. This highly sensitive and specific automated approach therefore may be a good screening tool and a supplementary adjunct to microscopy, to identify malaria in patients whose diagnosis may otherwise be delayed or missed.

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