Digital analysis of changes by *Plasmodium vivax* malaria in erythrocytes

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Blood samples of malaria patients (*n* = 30), selected based on the severity of parasitemia, were divided into low (LP), medium (MP) and high (HP) parasitemia, which represent increasing levels of the disease severity. Healthy subjects (*n* = 10) without any history of disease were selected as a control group. By processing of erythrocytes images their contours were obtained and from these the shape parameters area, perimeter and form factor were obtained. The gray level intensity was determined by scanning of erythrocyte along its largest diameter. A comparison of these with that of normal cells showed a significant change in shape parameters. The gray level intensity decreases with the increase of severity of the disease. The changes in shape parameters directly and gray level intensity variation inversely are correlated with the increase in parasite density due to the disease.

**Keywords:** Erythrocytes, Gray level intensity, *P. vivax* malaria, Shape descriptors

Malaria is an ancient disease that continues to cause enormous human morbidity and mortality¹. All the clinical symptoms of malaria, however, are a consequence of infection of human erythrocytes¹. The various stages of the growth of malarial parasites induce morphological and functional changes in infected erythrocytes. Some of these alterations in host cells appear to be involved in the development of malarial-related complications in the host². These include consumption and degradation of the erythrocyte proteins, appearance of knob-like protrusions in the membrane, electron dense material, clefts and vesicles in the cytoplasm²-⁴. With the increase of number of such erythrocytes the severity of malaria increases⁵,⁶.

The changes in membrane structure and composition of the cytoplasm as in hypercholesterolemia⁷ and hyperglycemia⁸ lead to the alteration in functional properties of erythrocytes⁹. The digital analysis of erythrocytes images, achieved by digitization followed by their on-line processing by computer, has helped in identifying the respective changes in the disease process⁷,⁸,¹⁰. Similar analysis has also been effective in the analysis of the influence of hypercholesterolemia on erythrocytes in hyperglycemia¹¹ and in the detection of *P.-falciparum* in blood smears¹². As during the invasion of malaria parasite the erythrocyte membrane and cytoplasm are subjected to changes², the parameters obtained by digital analysis of images could be implemented to correlate the cellular changes to the severity of malaria. This analysis forms the objective of the present work which is based on images of erythrocytes obtained from patients suffering from malaria caused by *P. vivax* parasite. Such a procedure may further help in developing a generalized procedure to correlate the cellular changes with various parameters.

**Materials and Methods**

**Sample preparation**—Blood samples were obtained from patients who tested positive for malaria parasite during their first visit to hospital. Thirty patients (*n*=30) were selected for the study and none of them has received any anti-malaria treatment at the time of this study. Ten age-matched healthy (*n*=10) subjects without any history of disease, as a control group, were selected. From the finger-tip blood, the thin and thick smears for erythrocyte morphology analysis and parasites density quantification were prepared and dried in the air, respectively.

**Malaria sample classification**—The thin blood smears were applied with Leishman’s stain for 2 min and later on washed to remove excess of stain for

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morphological features of erythrocytes. J.S.B. stain was applied for analysis of density features of malaria parasites in thick blood films. This stain is a fairly rapid staining method for the detection of malarial parasites clearer in both thick and thin smears. After drying, the slides were examined by the video-microscopic system (LEICA DM 2000, Germany), equipped with digital camera, using 100X objective of field size 1024×768 pixels. The magnification of the infected cells was same as that of normal erythrocytes. Based on the parasitized RBC count, ranging from 1–2, 3–5, and more than 5, these samples were classified in low (LP), medium (MP) and high (HP) parasite density, respectively. Out of 30 P. vivax malarial samples, 12, 10 and 8 were in the LP, MP and HP categories, respectively. From each view four sections were selected and from the count of infected cells an averaged value was obtained. This procedure was the same for all samples.

**Data acquisition and analysis**—The digitized images of erythrocytes, obtained by above procedure, were stored in the computer for further processing. From each category the images of cells were grabbed and processed to extract the contour by using the MATLAB, followed by the calculation of shape descriptors of each erythrocyte. The sequence of operation of this procedure is given in Fig. 1. For calculation of area, the contour of the cell was filled with pixels. By counting the number of pixels within the contour and multiplying the number of pixels with area of one pixel, the area \( A \) of the cell was determined. Similarly, for perimeter \( P \) estimation the number of pixels along the perimeter of the contour was counted and this number was multiplied by length of the pixel. Based on these parameters the form factor \( FF = \frac{P^2}{4\pi A} \) was calculated. This parameter is the measure of compactness or roundness of the cell and its variation indicates the deviation of the shape from that of a disc (FF for disc =1). For calibration of this procedure a circle of known diameter was taken. The form factor calculated from its area and perimeter by above procedure was equal to one.

For comparison of the variation of the gray level intensity in normal and low, medium and high parasitemia erythrocytes, these cells were scanned along their maximum width. The sequence of steps involved in this are: read the input image, grayscale conversion, median filtering, background subtraction and finally to perform horizontal scan to obtain the gray scale profile. For comparison purpose the normalized values of the horizontal distance at 0.5 of the full width for all types of cells was considered.

![Fig. 1—Sequence of procedures for determining the shape parameters of erythrocytes.](image1)

![Fig. 2— The microscopic images of (a) normal, and (b) malaria infected cells as obtained from blood smears.](image2)
Results

With the presence of parasite in erythrocyte its normal appearance is changed. The cells infected with *P. vivax* (Fig. 2) were enlarged, deformed, and when properly stained with Leishman’s stain, often showed stippling on the erythrocytes membrane known as “Schüffner’s dots”. All stages of the parasite were present in the peripheral circulation.

The erythrocytes that have become infected with malarial merozoites are subjected to a number of fundamental changes in their plasma membrane properties due to parasites development. An example of images of the background subtracted erythrocytes and their processed contours used for calculations of shape parameters are presented in Fig. 3. There was a change in the cytoplasm and the contour of erythrocytes with increase in parasitemia. Table 1 shows the variation of shape parameters in different types of infected cells and their comparison with that of control erythrocytes. The parameters area and perimeter increased with the increase of severity of the disease. The form factor, calculated by these parameters showed increase compared to that of control cells, which corresponds to significant increase compared to that of control erythrocytes.

From the background subtracted images of erythrocytes of control and different types of parasitemia the horizontal scans were obtained. Figure 4 shows an example of the variation in gray value as observed for various cells (Fig. 3), obtained by the horizontal scan of background subtracted cells. The gray level intensity was the maximum in the control cells, whereas, this decreased with the increase of parasitemia, which is attributed to the growth of parasite within the erythrocytes.

Table 1 shows the comparison of the gray level intensity variation in different parasitemia with that of control. A significant decrease in gray levels compared to that of control, at all stages of parasitemia, was observed.

Discussion

The present work describes the changes in erythrocyte shape due to *P. vivax* malaria under

<table>
<thead>
<tr>
<th>Samples</th>
<th>Area (µm²)</th>
<th>Perimeter (µm)</th>
<th>FF (P²/4πA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>55.47±3.2</td>
<td>25.40±1.71</td>
<td>0.97±0.11</td>
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<tr>
<td>Low parasitemia</td>
<td>62.79±3.81*</td>
<td>31.39±1.47*</td>
<td>1.26±0.14*</td>
</tr>
<tr>
<td>Medium parasitemia</td>
<td>64.99±2.98*</td>
<td>33.55±1.74*</td>
<td>1.35±0.22**</td>
</tr>
<tr>
<td>High parasitemia</td>
<td>66.36±3.14**</td>
<td>36.17±1.75**</td>
<td>1.57±0.38**</td>
</tr>
</tbody>
</table>

*P values: * < 0.05; ** < 0.01

Fig. 3—The background subtracted microscopic images of the normal (a) and malaria samples of low parasitemia (b), medium parasitemia (c) and high parasitemia (d) along with their respective extracted contours (e-h).

Fig. 4—Mean gray values of normal and malaria samples of low, medium and high parasitemia as measured along the largest horizontal distance between opposite ends of the erythrocyte membrane.
increasing disease severity conditions. The malarial samples were divided into three categories (LP, MP and HP) based on the parasitized red blood cell count in blood smear slides. Such a classification is appropriate as higher parasitemia level corresponds to greater severity of the infection and acute phase of malaria\(^{7}\).

During malarial infection the erythrocytes are subjected to biochemical, pathological and morphological changes\(^{14,6,12,13}\), which could lead to functional changes at the individual erythrocyte level\(^{14}\) as well as their cell–cell interaction\(^{15}\). Such changes have been recognized by light microscopy for a long time and have been called by various names such as Schuffner's dots, Maurer's clefts, Ziemann's stipplings and Stinton's and Mulligan's stipplings\(^{3,16}\). These host cell alterations appear to relate to the capability of malarial parasites to alter the properties of the erythrocyte and its membrane in order to export malarial proteins from the parasite to the host erythrocyte membrane. Although the significance of such changes is not clear, some of these alterations in host cells appear to be involved in the development of malarial-related complications\(^{9}\).

The present study with erythrocytes infected with parasite \(P.\) \(vivax\), measured at different degree of parasitemia shows a significant change in shape descriptors such as area, perimeter, and form factor with the increase of parasitemia. Erythrocyte deformability is an inherent property attributed to the size and shape of these cells. Any deviation from the discoidal shape alters the deformability of these cells. Hence variation in morphometric parameters such as surface area and perimeter may show the extent of changes in the flow properties of these cells\(^{7}\). These results show that the effect of \(P.\) \(vivax\) on erythrocyte deformability may also lead to microcirculatory complications similar to that as observed in malaria due to \(P.\) \(falciparum\)\(^{15,17}\) particularly in the HP category\(^2\). The regions where parasitemia cells are circulating could be maximally affected.

The analysis of internal activity of parasitized cells in comparison with normal cells was done by performing a scan along the maximum width of cells. According to Hommer et al.\(^{18}\), during its intra-erythrocytic cycle, the malaria parasite is located within the parasitophorous vacuole and, therefore, any molecular exchange with the red blood cell requires preliminary transfer across this membrane. In the first half of this intra-erythrocytic cycle, when the parasite is at the "ring" stage, there was no evidence of any effect of parasite development on the erythrocyte membrane, and all the known alterations were described in the second half of this cycle when the parasite was either at the late trophozoite stage or in the process of schizogony. The analysis of gray level intensity in both normal and malaria samples yield the information on the internal alterations. This is evident from the decrease in gray level intensity that there is an increase in parasitemia, as indicated by varying activity in different regions of erythrocytes.

In conclusion, the shape descriptors are very important for description of the changes which take place during the disease progression. An important parameter which is related to blood flow, the deformability of erythrocytes, could be assessed from the changes in these parameters. The time consuming process of determination of growth pattern of parasite was assessed by taking the scan along the larger width of the affected erythrocytes. Based on such studies an online process could further be developed for automatic detection of the changes induced by different malaria parasites.

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