Comparative Evaluation of Two Flowcytometric Analysers as Diagnostic Tools for the Automated Detection of Malaria

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Abstract. Goals. Automated flowcytometric analysers are used to analyse complete blood count (CBC) in most patients. They differentiate WBC depending on their size, internal granularity, and nuclear content by producing various scattergrams. In malaria, haemozoin pigment-containing cells (parasitized RBC and phagocytic cells) are able to depolarize the laser beam and therefore exhibit abnormal scattergram. Procedure. A study was conducted to evaluate the diagnostic utility and to determine the sensitivity and specificity of flowcytometric haematology analysers [Sysmex XE-2100 (System 1) and Cell-Dyn 3700 (System 2)] in comparison with the conventional microscopy (QBC, AO, Giemsa) and ICT (Immunochromatographic test) for detection of the malaria parasite. Results. Fifty-eight of 320 samples were found to be positive for malaria by conventional methods. Considering atypical scattergram, System 1 showed the sensitivity and specificity of 68.9% and 90.6%, respectively; whereas System 2 showed a sensitivity of 100% and specificity of 98.8%. Conclusion. System 2 (Cell-Dyn 3700) was found to be a highly sensitive and specific tool compared to System 1 and conventional methods. Hence, it may be preferred for automated detection of malaria in the blood samples of patients with a differential diagnosis of malaria and those who are subjected to CBC analysis.

Key Words: flowcytometric analyser, malaria, scattergram, microscopy, Quantitative buffy coat assay.

Abbreviation: AO Acridine Orange; QBC Quantitative buffy coat assay; CBC Complete blood count; WBC White blood cell; AbnS Abnormal scattergram; ICT Immunochromatographic test; DN Double neutrophil; DE Double eosinophil; PCM Purple coded event.

Introduction

Laboratory diagnosis of malaria is done by microscopy, serological tests, and molecular methods. Among these methods, microscopy [includes Giemsa stain, Acridine Orange (AO) and Quantitative Buffy Coat assay (QBC)] remains the most popular method and the gold standard for detection of malaria [1]. However, microscopy is time consuming, and its sensitivity varies depending on the technical expertise of the laboratory personnel. Recently, the use of flowcytometric analysers has been suggested in automated diagnosis of malaria. Flowcytometric analysers are used for complete blood count (CBC) in many patients during their routine haematological examination [2-4]. These analysers differentiate white blood cells (WBC) depending on their size, internal granularity (presence of granules inside cytoplasm), and nuclear content, producing various scattergrams on the monitor. In malaria patients, the haemozoin pigment within the parasitized red blood cells (RBCs containing trophozoite, schizont, and gametocyte) and the phagocytic cells (i.e. monocyte, macrophage, neutrophil) are able to depolarize the laser beam of the analyser, resulting in various abnormal scattergrams (AbnS)/abnormal events (for e.g. double neutrophil zone, double eosinophil zone, grey zone, decreased space between neutrophil and eosinophil zone in Sysmex flowcytometer, purple and green events above the threshold line in CellDyn flowcytometer) during CBC analysis.
Sysmex (Sysmex Corporation, Kobe, Japan) and Cell Dyn (Abbott Diagnostics, Santa Clara) are two prominent analysers with which much malarial research has been carried out. The DIFF graph of the Sysmex XE-2100 analyser consists of five parameters, showing lymphocytes, monocytes, neutrophils and basophils, and eosinophils, with a space between the neutrophil and eosinophil (Figure 1A) [5]. Because of the presence of haemozoin pigments, malaria patients produce AbnS in the neutrophil region/eosinophil region (e.g., double neutrophil zone, double eosinophil zone or decreased space between neutrophil and eosinophil/appear as grey zone (Figure 1 B-E) [2,3,6]. Cell Dyn uses laser scatter at various angles for granularity and lobularity (number of lobes of nucleus), thereby separating more granular cells (eosinophils) from the less granular (neutrophils, monocytes, lymphocytes, and basophils) [7]. Eosinophils are placed above the threshold line separating the rest of WBCs (Figure 2A). Detection of malarial haemozoin pigments in a Cell Dyn analyser was first reported by Mendelow and colleagues [8]. These are observed as purple and blue events above the threshold line and green events above the normal distribution of the eosinophil area (Figure 2B).

Few studies used these analysers for detection of malaria, but none of the studies has compared both of these analysers with the routine diagnostic methods that are available. Hence, the current study was designed to compare and evaluate the efficacy of these two automated analysers with the standard malaria diagnostic techniques, i.e., Giemsa, AO, QBC, and Immunochromatographic test (ICT).

Materials and Methods

The study was conducted in the Department of Microbiology over a five-month period. Three hundred and twenty blood samples were collected from patients with a clinical suspicion of malaria after receiving clearance from the Institutional Ethical Committee. All samples were tested for malaria by microscopy (Giemsa, AO, QBC), ICT (OptiMAL), and flowcytometric analysers [Sysmex-2100 (System 1), Cell Dyn-3700 (System 2)]. The laboratory findings were observed and confirmed by

![Figure 1. DIFF scattergram of Sysmex XE 2100 shows: A) normal, B) extended neutrophil with increased gap, C) double neutrophil, D) double eosinophil, and E) grey zone.](image-url)
three independent observers. Positive samples were analysed for parasitic load, haemoglobin, and platelet count. The AbnS in the DIFF graph in System 1 were noted as double neutrophil (DN), extended neutrophil, decreased space between neutrophil and eosinophil, grey zone, double eosinophil (DE), or any combination thereof. The granularity-versus-lobularity plots in System 2 were examined for purple events, blue events above the threshold line, and green events above the eosinophilic area. The presence of a single purple-coded event (PCM) was taken as positive for malaria. Cell populations forming AbnS in flowcytometers were analysed, and sensitivity and specificity for detection of malarial parasite was assessed by 2/2 table analysis. Five follow-up samples were collected 48 hours post-initiation of antimalarial treatment to observe any changes in the patterns.

Results

The study population included 320 clinically diagnosed malaria cases. Blood samples positive for any of the conventional methods, i.e., Giemsa/AO/QBC/ICT, were considered positive for malaria. Fifty-eight out of 320 blood samples were confirmed positive for malaria by any of the conventional techniques. Fifty-six cases were found to be positive for QBC, whereas 54 cases were confirmed by ICT (Table 1). *P. vivax* was the predominant species detected in 42 of 58 cases (72.4%), followed by *P. falciparum* in 13 of 58 (22.4%), and mixed infection of *P. vivax* and *P. falciparum* in 3 of 58 (5.1%) cases. Positive samples showed various morphological forms of parasite (early and late trophozoites, schizonts, gametocytes) under microscopic examination. WBCs (monocyte, macrophage, and neutrophil) containing malarial pigments were found in the buffy coat layer of QBC. QBC assay showed the highest sensitivity (96.5%) compared to other conventional techniques. Other techniques, like Giemsa and AO, were able to detect 36 (62.06%) and 37 (63.8%) malaria positive cases, respectively. However, the specificity for all the conventional methods was 100%.

Forty of 58 malaria-positive cases showed AbnS in System 1. It was also observed that most patients with low parasitaemia (≤200 parasites /µl) failed to develop Abns in System 1. An extended neutrophil zone with reduced space between neutrophil and eosinophil population was the most common abnormal pattern followed by grey zone, DN, DE, and/or a combination of DN and DE. Using criteria for atypical light depolarization (purple events, blue events, green events above the eosinophilic area) in a granularity vs. lobularity graph, System 2 could detect all 58 malaria-positive cases with a sensitivity of 100% and a specificity of 98.8%. Some samples showed red events in the area above the threshold line along with the other atypical events in the granularity vs. lobularity graph (Figure 2B).
Five blood samples were obtained from confirmed cases of \textit{P. vivax} and \textit{P. falciparum} 48 hours post-initiation of antimalarial treatment. These samples were checked for malaria parasites and analyzed for the AbnS in both the systems. All five samples had only intracellular haemozoin pigments within the WBCs without any morphological form of malaria parasite detected in QBC. The samples tested negative for malaria parasite with other conventional techniques i.e. Giemsa, AO, ICT. System 1 could detect three positive cases out of five while all cases were detected positive by System 2. The positive predictive value and negative predictive values of System 1 were found to be 90.6% and 59.7% (CI 55.4-80.4), respectively, in comparison to 95% and 100% (CI 93.8-100) for System 2.

### Discussion

Malaria is one of the most common public health problems in developing countries. During the erythrocytic phase, the malaria parasite digests the haemoglobin of red blood cells (RBC), releasing the haem group. This haem group converts to haemozoin pigments and is phagocytosed by the phagocytic cells. Unlike haemoglobin, haemozoin pigments have a tendency to depolarise laser light. Although malarial pigments have been detected by different microscopic methods, their presence in the parasitized RBCs as well as the phagocytic cells makes their detection in automated flowcytometric analysers possible [5,8-10].

Comparing the sensitivity and specificity of the conventional techniques, QBC was found to be the most sensitive method for detection of malaria parasite, followed by ICT. Fifty-six of 58 samples were positive, and the remaining two were negative for malaria by QBC. Considering abnormal scattergram, System 1 showed a sensitivity and specificity of 68.9% and 90.6%, respectively. In our study, we found System 1 had a sensitivity and specificity comparable to conventional microscopic techniques (Giemsa, AO). Our result was in agreement with other published literatures showing AbnS formed by neutrophil containing haemozoin pigments [2,3,6]. Most of the samples with low parasitaemia failed to produce AbnS in System 1. This may be why System 1 exhibited low sensitivity. The specificity of System 1 was found to be 90.6% in comparison to the conventional techniques. Extended neutrophil with decreased space between neutrophil and eosinophil was found to be the most common abnormal pattern, followed by grey zone leading to false positive results. It was found that normoblast, lymphoblast, and other immature granulocyte may contribute to the formation of AbnS because of their large size and high nucleic acid content.

System 2 showed a sensitivity of 100% in the detection of malaria. Various studies used System 2, which showed a high sensitivity (93-95%) for the detection of malaria [10,11]. Our results were in concurrence with these studies. Mendelow et al

<table>
<thead>
<tr>
<th>Methods</th>
<th>Positive</th>
<th>Negative</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Confidence Interval (CI %)</th>
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<tr>
<td>Giemsa</td>
<td>36</td>
<td>22</td>
<td>62.06</td>
<td>100</td>
<td>100</td>
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<td>48.4-74.5</td>
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<td>AO</td>
<td>37</td>
<td>21</td>
<td>63.8</td>
<td>100</td>
<td>100</td>
<td>92.5</td>
<td>50.1-74.5</td>
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<tr>
<td>QBC</td>
<td>56</td>
<td>02</td>
<td>96.5</td>
<td>100</td>
<td>100</td>
<td>99.2</td>
<td>88.1-99.6</td>
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<td>ICT</td>
<td>54</td>
<td>04</td>
<td>93.1</td>
<td>100</td>
<td>100</td>
<td>92.5</td>
<td>83.3-98.1</td>
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<tr>
<td>System 1</td>
<td>40</td>
<td>18</td>
<td>68.9</td>
<td>90.6</td>
<td>59.7</td>
<td>93.5</td>
<td>55.4-80.4</td>
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<tr>
<td>System 2</td>
<td>58</td>
<td>00</td>
<td>100</td>
<td>98.8</td>
<td>95</td>
<td>100</td>
<td>93.8-100</td>
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showed a racial variation in black and white patients in South Africa with sensitivity of 90% and 42%, respectively, for detection of malaria with System 2 [7]. However, some authors feel that the sensitivity of System 2 may vary depending on the severity of infection and host immunity [12-14]. The higher sensitivity of System 2 in the current study may be due to the criteria offered for positivity, such as the presence of a single purple coloured event above the threshold line. The specificity of System 2 was found to be 98.8% in comparison to the 100% specificity of conventional techniques, which is comparable to the specificity mentioned in the literature (95-98%) [10,11,4]. All the follow-up samples failed to be detected by any of the conventional methods except the QBC, where we found only WBCs containing pigment cells without any morphological forms of parasites. However, System 1 and 2 could be able to detect 3 and 5 follow-up cases of malaria, respectively. Low specificity in our study may be explained by the persistence of malarial pigments within WBCs in the circulation after successful treatment, which other techniques failed to detect.

**Conclusion**

To the best of our knowledge, this is the first study comparing of all the conventional techniques with both the flowcytometric analysers for detection of malaria. System 2 showed 100% sensitivity and 98.8% specificity. The current study suggests that flowcytometric analysis by System 2 (Cell-Dyn3700) for automated detection of malaria is a reliable tool, as compared to System 1 (Sysmex-2100) and other conventional techniques. It is rapid, easy to operate, and requires less technical expertise. Apart from parasitic forms it could also detect the haemozoin pigments within the phagocytic cells as a marker of malaria which is often missed by the conventional techniques. Hence, it may be used as a preferred diagnostic modality for detection of malaria in all blood samples with a differential diagnosis of malaria which are subjected to CBC analysis.

**References**