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**ORIGINAL ARTICLE**

# Variation in White Blood Cell Differential Fluorescence Scattergram: A Clue for Automated Malaria Diagnosis

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## ABSTRACT

**Background:** Malaria is an arthropod-borne disease caused by Plasmodium. Malaria is a significant public health challenge with about 3.3 billion human lives at risk in 97 countries with two hundred million estimated malaria cases and 600,000 fatalities. Timely diagnosis can prevent disease spread, loss of human lives, and its harmful impact on the economy therefore malaria diagnosis by automated hematology analyzer is of great value.

**Objective:** To assess identification of scattergram abnormalities in malaria samples and to compare its performance with Microscopy, RDTs & PCR.

**Study Design:** Descriptive and cross-sectional study

**Place and Duration of Study:** Department of Pathology, Liaquat University of Medical & Health Sciences Jamshoro, 06 months from May 2020 to October 2020.

**Methodology:** Eighty seven malaria suspected samples were included. CBC performed on Sysmex XN1000i followed by Microscopy of thin blood film, RDTs by using STANDARD™ Q Malaria P.f/Pan Ag Test, DNA extracted by QIAamp DNA mini kit, PCR performed by GenoAmp Real-time qPCR Malaria.

**Results:** The median age of patients was 24+13.93 years. WDF Scattergram abnormalities were observed in 95.4 %of samples with sensitivity of 97.6% and specificity of 100% respectively. PPV was 100% and NPV was 99.8%. 96.4% of patients were malaria positive by microscopy. ICT was positive in 96.6% of samples while PCR confirmed plasmodium presence in 97.7% samples.

**Conclusion:** Automated hematology analyzer provides high sensitivity and specificity for malaria diagnosis. The Sysmex XN-1000i can detect certain abnormalities in WDF scattergrams in malaria patients. As a result, the appearance of an abnormal WDF scattergram in a patient with a history of febrile illness, aids the pathologist in confirming a malaria diagnosis.

**Keywords:** Malaria, Plasmodium Vivax, Plasmodium Falciparum, WDF scattergram, ICT, RT qPCR

## INTRODUCTION

Malaria an arthropod-borne disease is caused by Plasmodium. In humans, it is the most prevalent parasitic infection.[1] Malaria remains a worldwide health concern with about 3.3 billion human lives at risk in 97 countries with 200 million estimated cases and 600,000 deaths.[2] The worldwide mortality rate for Plasmodium Vivax is documented as 0.1-1.6%.[3] According to WHO estimates 228 million malaria cases and 405,000 malaria-related deaths were reported in 2018.[4] Annually with 1 million estimated cases and 300,000 confirmed reported cases Pakistan has been included in the list of countries accounting for about >95% total regional malaria burden. The list includes Pakistan Afghanistan, Somalia, Sudan and Yemen.[5-7] According to the provincial breakdown of these confirmed reported cases during 2018 the highest number of cases was reported from KPK 32% and Sindh 32%, followed by Balochistan 17%, Tribal Districts 19% and Punjab 0.2%. In Sindh highest number of the malaria confirmed cases were reported from district Thatta then Sujawal and followed by Mirpurkhas.[6]

Laboratory, diagnosis of the malaria includes different techniques such as microscopic examination of stained thick and thin peripheral blood film, quantitative buffy coat, rapid diagnostic tests (RDTs) and molecular diagnostic methods i.e. Polymerase chain reaction.[9,10] In the diagnostic approach of Malaria the light microscopy is considered as gold standard.[11] There are many benefits of microscopy it helps in the identification and quantification of the disease causing agent and not only this it is cost effective and efficient approach.[12] The sensitivity of microscopy becomes questionable with low parasitaemia (≤50 parasitized erythrocytes/µl blood).[13] Rapid diagnostic test devices does not require enough training instead can be operated after minimal training and rely on immune chromatography which has no certain requirements such as usage of electricity, highly skilled laboratory staff and availability of equipment and reagents, due to this in the rural areas this diagnostic tool has greatly enhanced the availability of diagnostic services.[14] Variation with the sensitivity and specificity of these devices have been reported which occurs due to various possible reasons for instance high temperature, denaturation of antibodies due to humidity, difficulty in operating device and human error.[15] Another attractive and reliable approach to the diagnosis of Malaria is PCR. It has an edge over manual microscopy and sero-diagnosis by Rapid diagnostic test devices.[16] The molecular based diagnostic technique for the malarial diagnosis is highly sensitive and specific as it detects the molecular signature of the disease causing agent through nucleic acid amplification. Polymerase chain reaction equipment is high priced and its maintenance is costly too. For this reason, in low income setting the price of the reagents used for each test is prohibitive.[17]

For more than a decade, in the diagnosis of malaria, automated haematology analyzer has been of great value. After showing the first report in which the Cell-Dyn® (CD) analyzer (Abbott Diagnostics, Santa Clara, CA, USA) enabled the specific detection of malarial pigment in the WBC, the interest in automated haematology analyzers was renewed. This discovery led the basis of several studies confirming the potential of automated haematolgy analyzers in diagnosing malaria.[18] In this study WDF scattergram abnormalities by Sysmex XN 1000i were evaluated, which were considered a clue for malaria diagnosis.

## MATERIALS AND METHODS

This study was descriptive and cross-sectional conducted at Department of Pathology, Liaquat University of Medical & Health Sciences Jamshoro, 06 months from May 2020 to October 2020. Patients with history of febrile illness with all age group and unbiased gender were included and known cases of dengue were excluded. The study design was approved by the Ethical review committee of LUMHS.A written informed consent was acquired from patients and their legal guardians for participation during this study and publication of the findings.

Eighty seven malaria suspected samples were collected. The patients included 46 males and 33 females with median age of 24 years (range: 1–73 years) (Table.1). Peripheral venous blood

specimen of all the malaria suspected patients was drawn in EDTA vacutainer and stored at -38ºC till DNA isolation and subsequent analysis.

**Automated haematology analyzer:** The complete blood count (CBC) was performed on 6 part differential automated haematology analyzer Sysmex XN-1000i Japan following standard machine operating protocol. This automated haematology analyzer uses fluorescence and flow cytometry technology with a semiconductor laser to categorize white blood cells. It generates three forms of optical data about different cells. Side fluorescence light (SFL) is used to identify the complexity of internal structure of the cell such as granules and nuclear content, while forward scatter light (FSL) is used to indicate cell size.

**Microscopy:** Microscopic examination is considered as gold standard for this purpose thin peripheral blood smear was made stained with Leishman's stain and examined according to WHO standard guidelines by three independent microscopists.

**Immunochromatographic Rapid diagnostic test:** The immunochromatographic testing was performed by using STANDARD™ Q Malaria P.f/Pan Ag Test on fresh blood samples as per manufacturer's protocol. It detected both Plasmodium falciparum and Plasmodium vivax.

**DNA extraction:** Genomic DNA was extracted from peripheral blood by using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) consistent with the manufacturer's protocol.

**Polymerase chain reaction:** To avoid discordant results between the automated haematology analyzers and other diagnostic techniques such as (microscopy and rapid diagnostic tests) quantitative RT qPCR was performed by GenoAmp® Real-Time qPCR malaria according to standard operating protocol of the kit on the Applied Biosystems® QuantStudio® 5 Real-Time PCR System. The extracted DNA was amplified and quantified along with species identification. The results obtained from the internal quality control PCR were used to countercheck the results obtained from other diagnostic modalities.

The data was analyzed by using SPSS-22. Chi-square test was applied to compare categorical data and the p-value ≤0.05 was considered as statistically significant. Sensitivity and specificity of automated haematology analyzer (WDF scattergram abnormality) gold standard microscopy and ICT were calculated and compared with internal quality control PCR. The sensitivity was calculated as proportion of positive test results obtained among malaria suspected samples which were confirmed as malaria positive by PCR; the specificity was calculated as proportion of negative test results among malaria suspected samples which were confirmed as PCR negative. Positive and negative predictive values were also calculated as the proportion of true positive or true negative results among all samples which were confirmed as positive or negative by PCR, respectively.

## RESULTS

The median age of patients was 24 years. Males were in majority as 52.9% as compared to females 47.1% (Table 1). Generally, Sysmex XN 1000i is used for the evaluation of heamatological parameters and serves as a first line investigation in the workup of febrile patients. The study population tested for malaria revealed WDF scattergram abnormalities in 83/87 samples. This data obtained from the automated haematology analyzer was comparable to gold standard microscopy and ICT and internal quality control RT qPCR. Based on gold standard microscopy 84/87 samples were malaria positive with different stages of the plasmodium parasite examined which included ring form, trophozoite, schizont and gametocyte. 85/87 samples were confirmed as malaria positive by RT qPCR, including 80 samples positive for Plasmodium vivax, 04 samples positive for Plasmodium falciparum and 01 sample positive for mixed infection (PV and PF) (Table 2).

In 95.4% malaria suspected samples abnormal White blood cells differential fluorescence (WDF) scattergram with a supplementary purple cluster was noticed (Figs.1-3). 4.5% samples did not show any abnormality in the WDF scattergram among these 2.2% were true negative while 2.2% samples which didn't show distinctive purple color cluster were false negative. Among this one sample was containing ring form stage of Plasmodium vivax and other one with one gametocyte of Plasmodium vivax. WDF Scattergram abnormality had sensitivity of 97.6% and specificity of 100%. Microscopic examination of malaria suspected samples revealed 84/87 (96.5%) malaria positive cases. Out of 96.5%, 90% samples were infected with Plasmodium vivax, 4.5% with Plasmodium falciparum and 1.1% with mixed infection (PV and PF) while 3.4% samples were negative by microscopy among these 2.2% were true negative and while 1.1% sample was false negative missed by microscopy and detected by RT qPCR. Microscopy has sensitivity of 98.2% and specificity of 100%. Immunochromatographic test was performed by using STANDARD™ Q Malaria P.f/Pan Ag test. 84/87 (96.5%) samples were positive, among these 01 samples was false positive while 3.4% samples turned out to be negative on ICT. Among these 01 samples was true negative and 02 samples were false negative. ICT had a sensitivity of 97.65% and specificity of 50% (Table.3)

RT qPCR which was the internal quality control turned out to be the most sensitive and specific with sensitivity of 100% and specificity of 100%. It detected 85/87 malaria suspected samples among them 80 samples were infected by Plasmodium vivax, 04 by Plasmodium falciparum and 01 was mixed infection (PV and PF) while 2.2% samples were true negative. Plasmodium ovale, Plasmodium malariae and Plasmodium knowlesi were not found. PCR also detected a case of Plasmodium vivax gametocyte which was missed by gold standard microscopy. On comparison of all the diagnostic modalities the specificity and sensitivity of automated haematology analyzer was comparable with gold standard microscopy and the most sensitive RT qPCR (Table 3).

Table 1: Demographic data (n=87)

| Gender | No. | % |
|---|---|---|
| Male | 46 | 52.9 |
| Female | 31 | 47.1 |

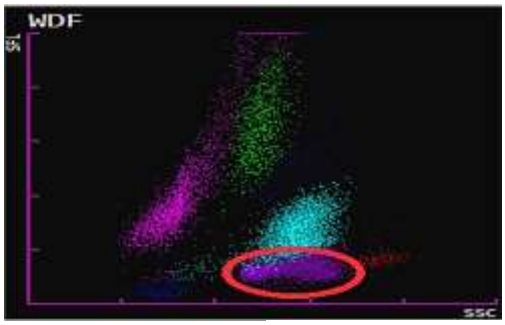
Table 2: Plasmodium species along with stages and automated hemtology analyzer data

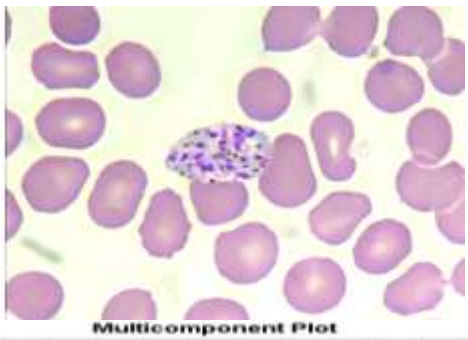
| Parasite stage | Plasmodium vivax = 80 | Plasmodium falciparum = 04 | Mixed infection (P.V & P.F) = 01 |
|---|---|---|---|
| Ring form | 1 | 1 | - |
| Trophozoite | 6 | - | - |
| Schizont | 28 | - | - |
| Gametocyte | 44 | 3 | 1 |
| WDF scattergram abnormality | 78 | 4 | 1 |

Table.3: Comparison of automated hematology analyzer with microscopy, ICT, and RT qPCR

| Variables | WDF Scattergram abnormality | Microscopy | STANDARD Q Malaria P.f/Pan Ag Test | RT qPCR |
|---|---|---|---|---|
| True positive samples | 83 | 84 | 83 | 85 |
| True negative samples | 2 | 2 | 1 | 2 |
| False positive samples | - | - | 1 | - |
| False negative samples | 2 | 1 | 2 | - |
| Sensitivity (95% CI) | 97.6% | 98.82% | 97.65% | 100% |
| Specificity (95% CI) | 100% | 100% | 50% | 100% |
| Positive predictive value | 100% | 100% | 12.13% | 100% |
| Negative predictive value | 99.8% | 99.92% | 99.67% | 100% |
| Accuracy (95% CI) | 99.84% | 99.92% | 53.14% | 100% |

WDF = White blood cells differential fluorescence WNR = White cell nucleated ICT = Immune chromatographic test RT qPCR = Real time polymerase chain reaction



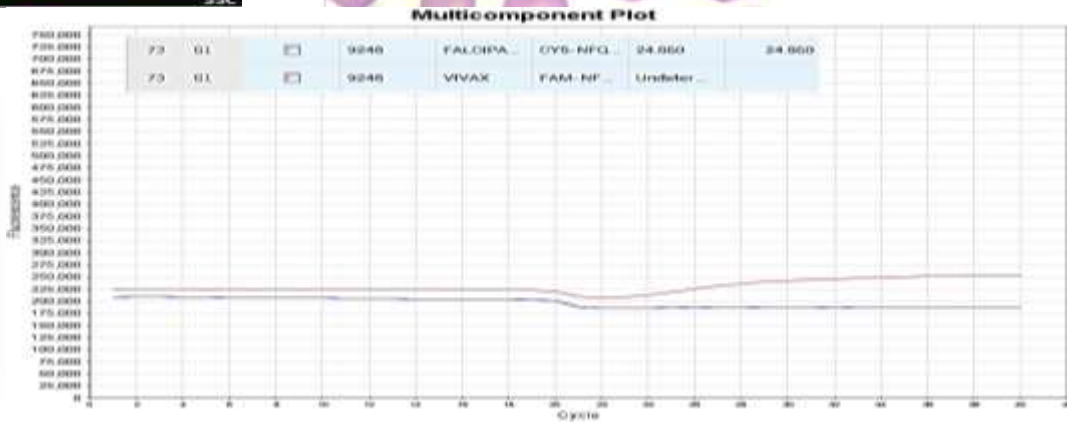
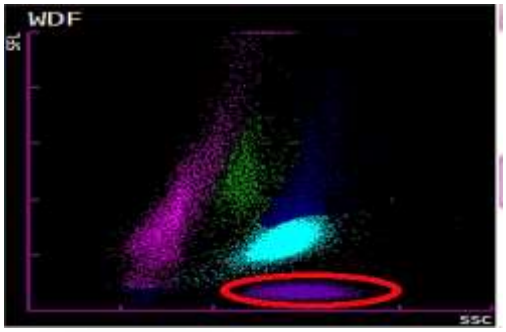
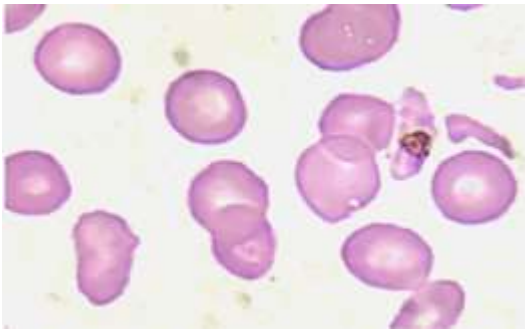




Fig 1: Plasmodium vivax detection.(1A) abnormal White blood cells Differential Fluorescence (WDF) scattergram illustrating additional distinctive purple color cluster (encircled area). (1B) on microscopic examination of peripheral blood film stained with Leishman's stain,a mature form of Plasmodium vivax (schizont) was observed. (1C) ICT positive Plasmodium vivax. (1D) RT qPCR confirming the presence of plasmodium vivax with Ct mean value 20,343



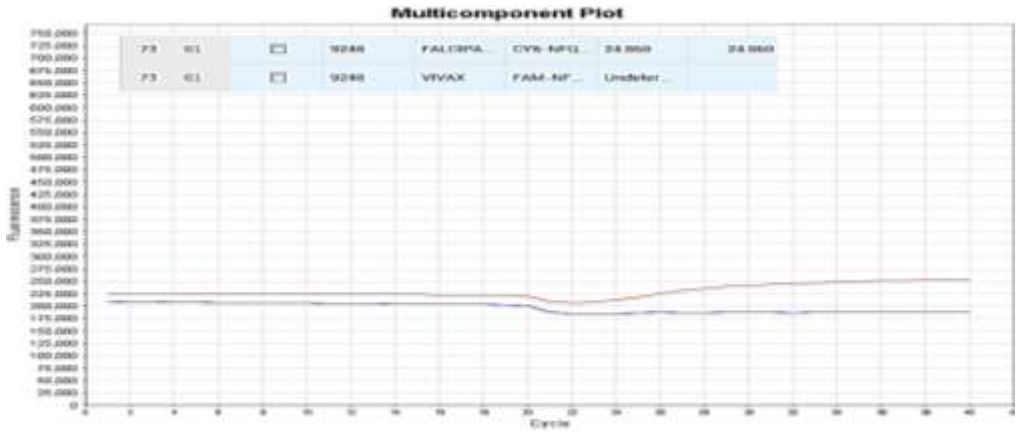




Fig 2: Plasmodium falciparum malaria detection.(2A) abnormal WDF scattergram showing supplementary purple color cluster (encircled area). (2B) mature stage of Plasmodium falciparum (gametocyte) was found on thin blood film. (2C) ICT positive plasmlodium falciparum. (2D) PCR confirmed plasmodium falciparum presence with Ct mean value 24,860.

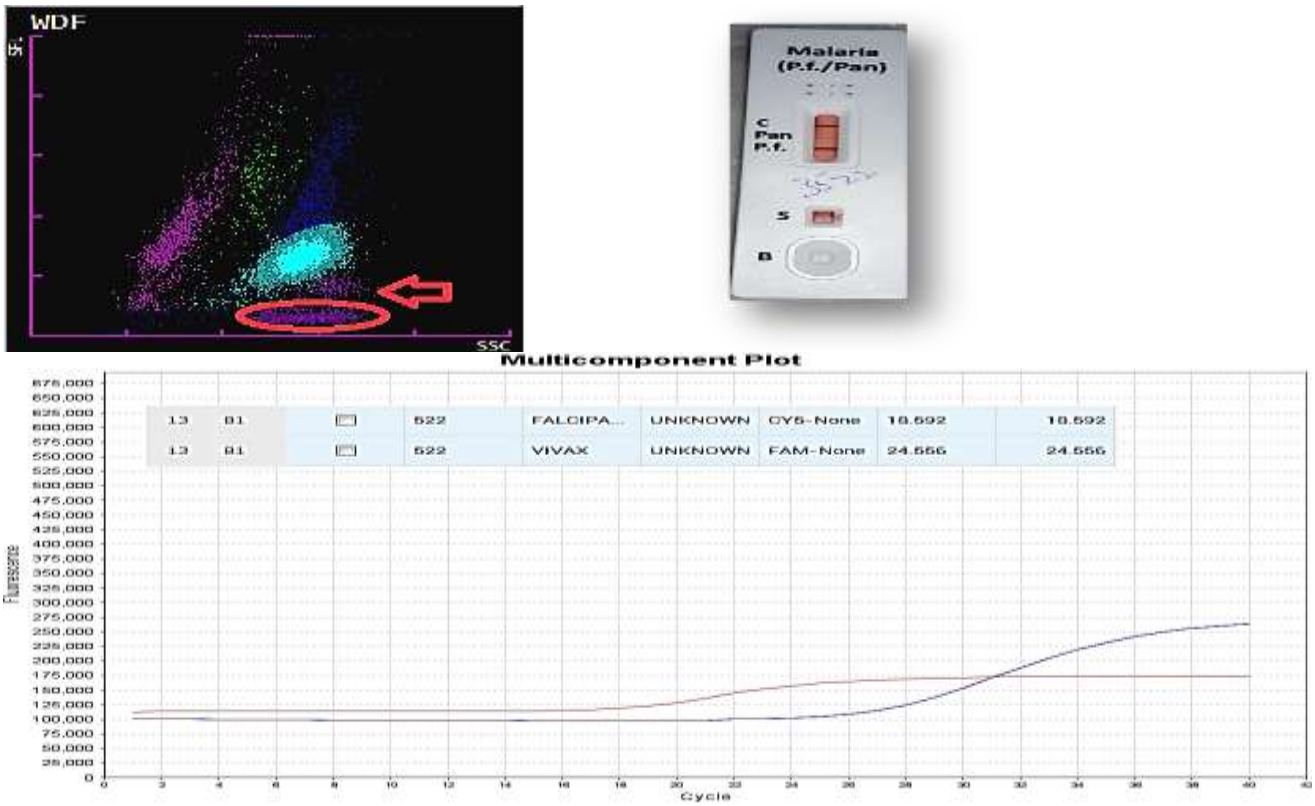



Fig 3: Mixed infection detection. (3A) Sysmex XN 1000i abnormal WDF scattergram with two distinctive purple color clusters.(3B) Mixed (P.vivax and P.falciparum) positive ICT. (3C) RT PCR positive for Mixed infection (P. falciparum $C_t$ mean 18,692 and P.vivax $C_t$ mean 24,556).

## DISCUSSION

In present research 87 malaria suspected samples were analyzed by flow-cytometry based hematology analyzer. The present study showed purple color cluster in WDF scattergram of 95.4% malaria samples mostly containing schizont and gametocytes. These findings are consistent with study from Ranoko et al[21] showed abnormal cell clusters in WDF scattergram in samples containing mature stages such as schizont and gametocyte.

In this study distinctive cluster on WDF scattergram was identified with sensitivity of 97.6% and specificity of 100%, PPV 100% and NPV 99.83%. 2.2% cases did not show the distinctive purple color cluster. These two samples include; one containing ring form of Plasmodium vivax and another one with low parasitaemia containing gametocyte of Plasmodium vivax. Similar findings were reported by another study by Ningombam et al[22] which reported particular patterns of clusters in both WDF & WNR scattergrams in malaria positive cases. Only two samples, one containing only a few ring forms of Plasmodium vivax and another one Plasmodium falciparum sample having very low parasitaemia with one gametocyte, these two samples did not show the abnormal cluster in the scattergram. Font et al[20] also reported presence of distinctive cluster in the scattergram in malaria suspected sample with negative microscopy and RDT.

There are five species of malaria. In this study Plasmodium vivax was the commonest specie 92% followed by Plasmodium falciparum 4.0% and then mixed infection 1.0%. This is in line with other studies showing P.vivax, P.falciparum and mixed species prevalent within Pakistan with higher incidence of P.vivax as compared to other two species.[23] In our study microscopic examination had a sensitivity of 98.8% and specificity of 100%. This is comparable with a study by Rodulfo et al[24], they reported higher sensitivity of microscopy 95.7% and specificity of 98%.

In our study antigen based rapid diagnostic test performed by STANDARD Q Malaria P.f/Pan Ag test was positive in 96.6% cases with sensitivity 97.65% and specificity 50%. This is comparable with study conducted by Ahmed et al[25] revealing higher sensitivity 97.9% and low specificity 2.03% of the STANDARD Q Malaria P.f/Pan Ag test.

Molecular diagnostic technique PCR i.e. nucleic acid based detection of plasmodium species is highly sensitive and specific as compared to the gold standard microscopic examination. Naeem et al[26] reported superiority of RT PCR as it detected all the cases positive as well as negative on microscopy. Mukry SN et al[3] reported a negative sample by microscopy detected as Plasmodium vivax by species specific qualitative PCR. During this study similar findings were observed, RT PCR detected malaria parasite in 97.7% samples out of them vivax was commonest in 92% patients, falciparum was in 4.5% patients, 1.1% were mixed infection. One case negative on microscopy was detected as Plasmodium vivax by RT PCR.

## CONCLUSION

The automated hematology analyzer Sysmex XN 1000i is the ideal diagnostic modality for the early detection of malaria in patients with or without clinical presentation. It provides high sensitivity and specificity for malaria diagnosis. Utilization of automated hematology analyzer for malaria diagnosis will not immediately replace the gold standard microscopy and RDTs but certainly it should be in consideration as an adjunctive diagnostic modality.

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