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Research Article

Malaria detection by automation: The Manipal experience

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Abstract: Diagnosis of malaria has always been a diagnostic challenge in endemic areas. For many centuries microscopy based diagnosis has been a standard method for routine diagnosis of malaria. And still this is a routine diagnostic method used in low endemic areas which allows species identification. In endemic countries like India microscopy based diagnosis of malaria is still used and despite the presence of expert microscopists, laboratory misdiagnosis of malaria is still a problem. This may be due to immense work load, limited resources and manpower. The aim of the study was to confirm utility of the formula developed by *Briggs et al* and evaluate the feasibility of rapid diagnosis of malaria by using CBC data and *malaria factor* derived from standard deviation (SD) values of lymphocyte and monocyte by using haematology counter Beckman - Coulter series LH 750 and 755^{TM}. Three hundred and ten cases and controls were selected from samples sent to clinical lab for evaluation of fever and for routine examination. All cases and controls were scrutinised for *malaria factor*, thrombocytopenia, monocytosis, mean monocyte volume and pseudoeosinophilia. At cutoff value of 3.4, 97% sensitivity and 89% specificity was obtained. Detection of malaria by automated hematology counters my replace current screening methods for detection of malaria in future, but need of extensive study in different population is need for validation of this method.
Keywords: Malaria, Automation, Malaria Factor, VCS technology, Malaria pigment, Monocyte.

INTRODUCTION

India is an endemic zone for malaria with approximate of 1.6 million cases reported each year and 15,000 deaths in 2006[1]. This number has been contested by researchers such as Hay *et al* [2] and Dhingra *et al* [3] who predicted the incidence and mortality is much higher. Diagnosis of malaria has always been a diagnostic challenge, especially in developing countries like India due to a high workload, lack of resources and affordability. Hence rapid diagnostic methods are quite essential to prevent mortality.

Light microscopy techniques are considered gold standard for diagnosis of malaria. While speciation of the parasite is possible on a peripheral smear, it puts an additional load on pathologists to screen a vast number of smears. Current guidelines suggest the use of thick and thin smears stained with leishman or gimsa stain for diagnosis, speciation and quantification of malaria parasite. It involve screening the slides for malaria parasite in 200 fields under oil immersion (X100 objective), which usually takes 20-40 min. Quantification of parasite (parasite load) is performed by counting ring forms and schizonts of P.vivax , ring forms and gametocytes of P.falciparum using thin smears under oil immersion in minimum of 1000 red blood cells [6, 7] . Some laboratories uses QBC as screening test, but the capital and test costs are higher and not affordable by commoner in developing countries like India. Although few laboratories use supplementary tests like immunochromatograhic tests to detect malarial antigen along with thick and thin smear; it is not without disadvantages. These include 1) false positive in high WBC counts or in leukemic patients, 2) persisting histidine rich protein-2 for days after treatment, 3) except for P.vivax and P.falciparum other species cannot be identified, 4) quantification not possible, 5) rarely prozone effect [7, 8].

Since the advent of flow cytometry based automated haematology analysers; diagnosis of malaria has taken rapid strides and have become an adjuvant tool in routine investigative work up of patients with acute febrile illness. Thus, a need for more sensitive and cost-effective method has interested researchers in past.

Beckman Coulter series that is LH 750 and 785^{TM} analyzers uses volume, conductivity and scatter (VCS) technology i.e., impedance for cell volume,

radiofrequency for conductivity and flow cytometry based helium neon laser light scatter analysis (side scatter) for cellular granularity & nuclear lobularity for compete blood analysis [4].

Malarial parasite infestation is known to recruit and increase the number of blood monocytes and lymphocytes, with their activation and increase in their size [5]. Recently many studies conducted in USA and a large joint study conducted in England and South Africa, in which coulter Gen. S haematology analyser was used to differentiate between malaria positive and malaria negative cases. This was done by using a Discriminant factor (Malaria Factor) derived from differences in standard deviation (SD) of volume of lymphocytes and monocytes [5, 10] .

Malaria is known to cause haematological aberrations including anaemia, thrombocytopenia and monocytosis. Additionally, hemozoin (malarial pigment) is produced by malarial parasite after detoxification of free heme produced during haemoglobin digestion, and is phagocytosed by granulocytes and monocytes. These cells produce different scatter patterns in automated cell analyzers. Thus, we decided to conduct a study by using VCS technology for rapid diagnosis of malaria by using a discriminant factor using haematological data and research population data (RPD) since we inhabit an area which is regarded endemic for *Plasmodium* species (South Karnataka, udupi).

MATERIALS AND METHODS

A cross sectional case-control study was conducted for period of three months in the Clinical Laboratory and Haematology division, after institutional ethical committee clearance and approval. Study was aimed to confirm utility of the formula developed by *Briggs et al* and evaluate the feasibility of rapid diagnosis of malaria by using CBC data and *malaria factor* derived from standard deviation (SD) values of lymphocyte and monocyte by using haematology counter Beckman - Coulter series LH 750 and 755™ . Cases were selected from samples sent to clinical laboratory for quantitative buffy coat (QBC) analysis for detection of malaria i.e., malaria positive cases. Two types of controls were selected: samples of patients with fever but malaria negative by QBC and samples of patients who were afebrile and had come for routine check-up. Samples were analysed in automated haematology counter Beckman - Coulter LH 750 and 755™ series. The complete blood count (CBC) and RPD were retrieved using the machine software. There was no direct interaction with the patient, in other words samples were randomly selected which were sent to our clinical laboratory for work up.

90 cases of malaria and 220 controls were scrutinized using following parameters

a) Malaria factor = (SD volume of lymphocytes × SD volume of monocytes)/100
b) Thrombocytopenia
c) Monocytosis
d) Mean volume of monocytes
e) Pseudoeosinophilia

Statistical Analysis

CBC counts, differential counts, standard deviation (SD) of volume of the lymphocytes and monocytes were recorded for all samples. Malaria factor was calculated by following formula developed by *Briggs el al*.

Malaria factor = (SD volume of lymphocytes X SD volume of monocytes) / 100.

Data was recorded on Microsoft excel ™ version 2007 spread sheet and analysed by using SPSS .20 version (Chicago, Illinois, USA) IBM. Statistical analysis was done and receiver Operative Characteristic (ROC) analysis was done to determine satisfactory cut-off value for detection of malaria in samples.

RESULTS

Among 130 clinically suspected malaria cases, 80 were positive for *Plasmodium* vivax, 10 were positive for *Plasmodium* falciparum and 40 were negative for malaria by quantitative buffy coat method (QBC). 180 controls were selected from non febrile patients coming for routine work up.

On comparison of samples from all three groups, it was found that mean SD volume of lymphocytes and mean SD volume of monocytes are significantly higher among malaria positive cases than control population. Similarly, mean platelet count was significantly lower in malaria positive cases, but in controls it was found to be normal. Mean volume of monocytes was found high in both cases and control population with fever, but it was significantly lower in controls without fever. Eosinophil count and mean platelet volume were found statistically not significant in our study population. No significant raise in eosinophil count were found in our study. The values of above mentioned parameters are summarised in table 1.

On ROC analysis for Malaria Factor, at the cut-off value of 3.4, sensitivity of 97% and specificity of 89% was obtained (Figure 1).

By using the malaria factor at a cut-off value of 3.4, 86 out of 90 cases were found to be positive for malaria by the automated haematology analyser.

The 23 false positive samples were from group of controls who had fever but up on workup for malaria, that is QBC was found negative. 3 among 4 false positive cases were seen in cases with P.vivax infection

and one was attributed to low event in the automated hematology analyser.

True positive rate, true negative rate, false negative rate and false negative rate were used to calculate positive predictive value (PPV) and negative predictive value (NPV) (table 2). A PPV of 78.8% and NPV of 98 % were obtained.

Average cut off for malaria factor in cases of P.vivax was found to be 4.8 and for P.falciparum were 5.8. There was no significant correlation between level of parasitemia and malaria factor.

But when malaria factor at cut off of ≥ 3.4 was combined along with platelet count of > 150 X 10^3 /cumm, mean monocyte volume of < 180 fl, SD volume of monocyte of < 25 and no abnormality in scatter gram; the negative predictive value increased significantly.

Table-1: C**omparison of mean values of haematological parameters which showed significant changes between cases and controls:**

	Mean SD volume of lymphocytes	**Mean SD volume of monocytes**	**Mean Platelet count (x10^3)**	**Mean volume of monocytes (fl)**	**Eosinophil count (%)**
Malaria positive cases (n=90)	19.57	25.67	93.2	180	0.99
Malaria negative controls – with fever (n=40)	16.3	22.64	211.5	172	1.5
Malaria negative controls – without fever (n= 180)	13.79	17.42	253.1	152	3

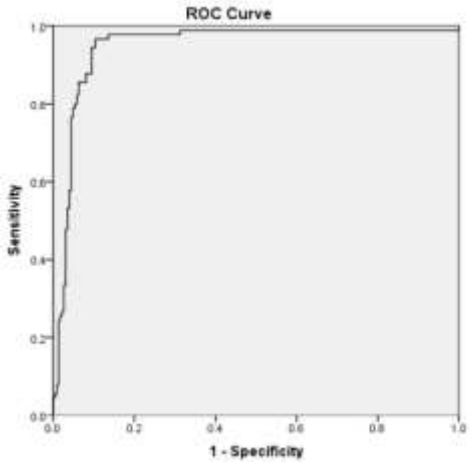

Fig-1: A receiver operating curve for malaria factor, the area under the curve 0.946. Cut-off value for malaria factor was 3.4; sensitivity of 97%; specificity of 89%.

Table-2: True positive, true negatives, false positive and false negative cases in study population (n=310).

Malaria factor at a cut-off value of 3.4	**Number of cases and controls**
True positives	86 (95.6%)
True negatives	197 (89.5%)
False positives	23 (10.5%)
False negatives	4 (4.4%)

DISCUSSION

Microscopic examination of thick and thin blood smears are regarded "gold standard" investigations for detection of malaria. But these procedures are time-consuming and disadvantageous when a very low level of parasitemia is encountered [7]. Many methods have been introduced till date and one among accepted methods is QBC i.e., Quantitative buffy coat analysis for detection of malaria parasite by various labs as an initial screening technique. The disadvantage of QBC is high cost of equipment and consumables and need of trained personal. In

developing countries like India, not all laboratories can provide this service. And sensitivity of immunological methods for detection of malaria remains a problem.

After the initial breakthrough report, showing the utility of an automated haematology analyzer for detection of malaria pigment in leucocytes by *Mendelow et al,* there has been a series of studies confirming the potential of automated haematology analyzers in the diagnosis of malaria during routine CBC analysis for patients with fever [6,13,16,17]. *Hanscheid T* discussed various methods of diagnosing malaria by using VCS technology which include detection of malaria by detecting malaria pigment in routine hematology analysers and flow cytometry based analyser for detection of malaria [6]. *Jain, et al* suggested that an increase in number of monocytes and its volume along with increase in volume of lymphocytes and appearance of dots in eosinophil area are highly specific for malaria infection [9].

Briggs, et al suggested the use of standard deviation (SD) of volume of lymphocytes and monocytes to flag the possible presence of malarial parasites due to presence of reactive lymphocytes and histiocytic monocytes with engulfed malarial pigment in infected patients [5,12]. Observations of these findings lead to development of an algorithm that is Discriminant factor or Malaria factor. By using malaria factor at cut-off value of > 3.7; *Briggs, et al* obtained sensitivity of 98% and specificity of 94% [5].

In our study, Malaria Factor at cut-off value of > 3.4 was found to be 97% sensitive and 89% specific to detect the malarial parasite ($\prod$= 0.95). False negative rate of 10.5% in our study was seen exclusively in patient with fever but negative for malaria by QBC and found to be associated with bacterial infection or viral infection, due to increase in volumes of both lymphocytes and monocytes. False positive rate of 4.4 % was seen in patients with P.vivax infection, one of which was due to a low event and other three cases were due to random error. No false negative rate was seen with P.falciparum infection.

But when malaria factor of $\geq$ 3.4 combined along with platelet count of > 150 x 10^3 /cu.mm, mean monocyte volume of < 180 fl, SD volume of monocyte of < 25 and no abnormality in scatter gram ; the negative predictive value increased significantly.

Relationship between variations of platelet count in malaria positive cases was inversely proportional. That is decrease in platelet count was seen in malaria positive cases than in malaria negative cases with fever and without fever. Reduction in platelet count in malaria positive cases was statistically significant between three groups. Absolute eosinophil count was not increased in any of groups. Statistical analysis for eosinophil count was not done for any of groups.

Using the above-mentioned algorithm, if the automated haematology analysers are calibrated for "suspect flags", unsuspected cases of malaria can be diagnosed early, thus decreasing the disease burden and mortality. With a negative predictive value of 98 % in our study, an absence of flag could also possibly rule out malarial infection in patients with acute febrile illness. Such a technology could supplant the "gold standard" tests.

CONCLUSIONS

The present study demonstrates the usefulness of fully automated haematology analyzers as a potential baseline diagnostic tool for rapid diagnosis of malaria in patients with acute febrile illness. The VCS-based algorithm for the detection of malaria was found to be highly reliable. The method is easy and requires lesser technical expertise. Future studies could throw more light on species identification and cases with low parasitemia.

Limitations

Our study is not without its limitations. One: we were blinded to the nature and demographic cases and controls. Two: the extrapolation of our findings in paediatric population may be limited, since paediatric blood cells are physiologically larger.

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