

Original Article

COMPARATIVE MALARIA DIAGNOSIS BY USING LH750 BECKMAN COULTER AUTOMATED ANALYSER IN INDIA: A CROSS SECTIONAL CASE CONTROL STUDY

Running Title: Malaria Diagnosis using VCS data by Automation

Abstract

Objective: To study the VCS pattern in patients with malaria and compare it with patients

with having fever who were malaria parasite negative for malarial parasite (*P.falciparum*,
vivax, *malariae* and *ovale*) and normal individuals and to determine whether a further
adaptation of the discriminant factor is needed using additional parameters from the VCS
data analysis using Beckmann Coulter LH 750.

Design: Cross sectional Case control study

Setting: Clinical Laboratory and Haematology Department, KMC, India

Patient(s): Ninety-three Malaria positive samples and fifty malaria negative samples by
QBC method comprised the Case and hundred healthy adult samples formed the Control
group.

Intervention(s): All blood samples were analysed using LH750 Beckman Coulter for VCS
data of the neutrophils, lymphocytes, monocytes and eosinophils and compared between
Malaria positive, Malaria negative and Control Group.

Main Outcome measure: Discriminant Factor

Comment [DBM1]: Title of the study changes as suggested

Comment [DBM2]: All four species were studied and proved negative.

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Comment [DBM3]: Institute ethical committee approval was taken and the study started only after obtaining the same. It is mentioned in line 72 under heading materials and methods. Informed consent was taken from the patients. Blood analysis was done as part of work up. We had explained the Data will be used for our study.

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Result(s): The difference in SD We used the significant difference in the SD volume of the lymphocyte and monocyte among malaria positive and negative were statistically significant to differentiate the malaria positive from malaria negative. By using logistic regression, the “Malaria factor” is defined. ROC analysis showed using By using a cut-off value of 2.7 as malaria factor derived from SD volume, specificity of 92.5 and sensitivity of 83.7 with a PPV of 88.6% and NPV of 89.1% were obtained whereas, specificity and sensitivity obtained using SD conductivity (91 and 75.5 respectively) and SD scatter (68.7 and 57.1 respectively) were significantly less.

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Conclusion(s): A “suspect malaria” flag could be generated on the analyser, allowing the detection of cases of unsuspected malaria and therefore, early diagnosis of the disease with the potential of reducing the possibility of serious complications however this is not a confirmative test.

Comment [DBM4]: No sir I have not compared with RPA technique. It was not available in our hospital during the study period.

Key words: Malaria; Malaria Diagnosis; Automated analyser; Coulter; Malaria Factor.

Introduction

Malaria is a major health problem with over 40% of world’s population exposed to varying degree of malaria risk. India is endemic to malaria with a case load of 1.49million in 2010^[1]. WHO estimates 216million cases with 445000 deaths worldwide in 2006^[2]. Cornerstones in global malaria control strategy is effective disease management by prompt and accurate diagnosis. Earliest symptoms of malaria are nonspecific and hence the clinical diagnosis is challenging and not reliable as it overlaps with most of the viral diseases and febrile illnesses^[3,4].

Despite good sensitivity and specificity, all malaria tests including the new ones have the inherent disadvantage that they have to be requested explicitly and lack of clinical suspicion

have been reported as one of the main reasons for misdiagnosis^[5]. Parasitological diagnosis of malaria is by Giemsa-stained Thick blood Film (GTF) that help in speciation and diagnosis. Other methods like Quantitative Buffy Coat (QBC), and Rapid Diagnostic Tests (RDT), and PCR are not highly sensitive and thus not reliable in detection in low-density malaria infections. Recombinase polymerase amplification (RPA) is a new mode of diagnosis with high specificity. However, it is costly and is not being used extensively. Hence, the need for a sensitive and reliable test using laboratory technology.

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Automated analysers uses VCS technology to quantify WBCs, it can also detect the infected hemozoin ingested WBC by the difference in volume conductivity and scatter as this has a different scatter plot and this can be used as a screening method especially in endemic area^[6,7]. Malaria detection in previous studies by automation used a discriminant factor derived from differences in standard deviation (SD) of volume of lymphocytes and monocytes^[8,9]. Factor was different in each study and varied with each species of plasmodium. We analysed the VCS pattern in patients with malaria and compared with malaria parasite negative fever patients and normal population to deduce a Malaria factor that can be used in Indian population to detect the possibility of infection. This study gains importance by being conducted in South Karnataka, which is Malaria endemic.

Objectives:

1. To study the VCS pattern in patients with malaria and compare it with patients with fever who were malaria parasite negative and with normal individuals.
2. To evaluate the feasibility of automated malaria detection using a defined Discriminant Factor and to determine whether a further adaptation of the discriminant factor is needed using additional parameters from the VCS data analysis using Beckmann Coulter LH 750.

Methods

The study area (Udupi District) is endemic for malaria infection with 1188 cases reported in 2010, of which 194 were *P. falciparum* infection. Our study was a Cross-sectional Case control study with the blood samples collected from patients attending Hospital with fever or clinically suspected malaria during the study period 2 months from July 2011 to August 2011. The Ethical Committee had approved the study IEC231/2011 of Institute approved study. Samples collected were from adult individuals were grouped into three groups.

- Group 1: Patient with fever and diagnosed as Malarial Parasite positive by fluorescent method (Quantitative buffy coat) and/or Immunochromatography. Ninety-three patient sample during the study period formed the study group.
- Group 2: Fifty samples of patient with fever and diagnosed as Malarial Parasite negative by fluorescent method (Quantitative buffy coat) and/or Immunochromatography.
- Group 3: Hundred samples from apparently healthy adults in whom all CBC parameters were within reference range and from voluntary blood donors formed the Control group.
- Patients with previously diagnosed systemic diseases presenting with fever were not included in the study. We ensured precision of VCS data since we run two level controls for CBC & differential count twice daily on Beckman Coulter LH 750.

The quantitative buffy coat method (QBC) combines an acridine orange coated capillary tube, an internal float, and separates layers of Platelets, WBCs and RBCs using

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Comment [DBM5]: All patients who attended hospital and diagnosed as malaria positive during the 2 months study period were recruited into the study. Malaria negative sample and normal sample size were not calculated from any study.

centrifugation. Parasites concentrate below the buffy coat and appear in the upper layer of RBCs. The parasites can be seen when the tube is examined under a UV light source^[10].

Protocol

We collected all peripheral blood samples in K₃EDTA tubes and analysed within 2 hours using LH750 Beckman Coulter for VCS data. We recorded the VCS data of the neutrophils, lymphocytes, monocytes and eosinophils and compared between Malaria positive, Malaria negative and Control Group. In Malaria positive samples, there was volume heterogeneity in lymphocytes and monocytes.

Variables

Outcome variable is Malaria detection. Dependant variables are Volume, conductivity and scatter data. Confounding factors are age and gender.

Study size

All malaria positive patient samples during the study period July 2011 to August 2011 (2months), which was 93 cases comprised the study group. Fifty Malaria negative samples confirmed by QBC/Immunochromatography and hundred samples from apparently healthy individuals formed the control group.

Statistical Analysis

Data was entered and analysed using SPSS (version 15, SPSS South Asia Bangalore).The continuous variables were analysed using mean and standard deviation. Comparison of the continuous variables between the patient and normal subjects were done using one-way analysis of variance. By logistic regression Malaria factor was calculated as:

116 Malaria factor = $\exp(-13.880 + (0.246 \times \text{lymphocyte_SD}) + (0.475 \times \text{monocyte_SD}))$

117

118 Receiver operating characteristic (ROC) analysis was performed on the malaria factor to
119 determine whether a satisfactory cut-off value could be established for the detection of
120 malaria in a blood sample. The results of the SD volume, conductivity & scatter of the
121 neutrophils, lymphocytes, monocytes & eosinophils were analysed by using the Kruskal
122 Wallis test to determine any statistical differences between three groups.

Comment [DBM6]: Sir, with all due respect I couldn't understand what was inferred by your comment. Thank you

123 Results

124 During the 2 months study period, we found 93 cases to be malaria positive among the
125 suspected cases. All malaria positive cases out of the total 243 samples, had volume
126 heterogeneity in lymphocytes and monocytes as compared to malaria negative cases and
127 normal samples. Table 1 shows the range of Standard deviation (SD) of lymphocyte and
128 monocyte observed in the study. The difference in SD for the variables among all 3 groups
129 were statistically significant ($p = 0.000$) for SD volume of neutrophil, lymphocytes,
130 monocytes, SD conductivity of neutrophil, lymphocyte, monocyte, eosinophil, SD scatter of
131 neutrophil and lymphocyte as shown in Table 2.

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Comment [DBM7]: All p values were 0.000

132 On comparing the significant difference in mean volume, conductivity and scatter
133 between malaria positive, malaria negative and control groups, SD volume of Monocytes and
134 Lymphocytes among malaria positive cases were significantly different from negative and
135 control group (Table 3). Malaria factor was calculated using logistic regression. We did ROC
136 analysis for the malaria factor thus calculated from the equation using SD of Volume,
137 Conductivity and Scatter. Figures 1 shows the ROC curve of Malaria factor calculated from

138 the SD volume, Conductivity and Scatter. Table 4 summarizes the findings of the ROC
139 analysis between volume, conductivity and scatter.

140 True positive, true negative, false positive and false-negative results obtained in Volume,
141 Conductivity and Scatter are given in Table 5. These figures were used to calculate the
142 positive predictive value (PPV) and negative predictive value (NPV) for this study. From the
143 findings, the Malaria Factor obtained using the SD volume difference in lymphocyte and
144 monocyte has the highest specificity and sensitivity of 92.5 and 83.7 respectively with a PPV
145 of 88.6% and NPV of 89.1%.

146 Discussion

147 Malaria is not only a health issue for the developing country but an economic burden. It is
148 one of the most common parasitic disease in the world. Morbidity of the disease is attributed
149 to delay in diagnosis, the mainstay of which is clinical suspicion. The “gold standard”
150 diagnostic modality for malaria is still Giemsa-stained Thick blood Film (GTF), microscopic
151 examination of stained thick and thin blood films. Disadvantage being it is difficult, time-
152 consuming and low levels of parasitemia may be missed^[11]

153 Quantitative Buffy Coat (QBC) technique is used as a backup to blood films and to
154 simplify malaria diagnosis^[12]. The overall sensitivity rate for QBC in a study by Adeoye et
155 al^[13] was 55.9% when compared with GTF. Sensitivity of QBC and GTF reduces further with
156 samples having low parasite density. QBC test cannot differentiate accurately between
157 different Plasmodium species but with the GTF. In spite of the speed and simplicity of QBC
158 technique, it cannot be considered an acceptable alternative to GTF under routine clinical
159 laboratory situation. The disadvantages of this method are the high cost of the equipment,
160 nonspecific nature of stain and consumables. Many rapid methods are present in the market
161 with variable detection rate, which still needs clinical suspicion to be asked for. High false

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Comment [DBM8]: No sir I have not compared the factors like time involved in obtaining microscopy and automated analyzer. We have not looked for the blood level of parasites to correlate with the findings.

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positivity in high WBC counts or in leukemic patients, persisting histidine rich proteins for days after treatment, inability to detect P.ovale and malariae, prozone effect and inability to quantify adds as the disadvantage for these methods^[14]. Polymerase chain reaction has been proven to be sensitive in the diagnosis of all four species of malaria. However, it is expensive and impractical in the routine diagnosis of malaria.

Sensitivities of immunologic methods for detecting malaria remain a problem. In non-endemic areas for malaria, the parasitemia is often very lower than the detection capability of devices involving capture of HRP-2 and pLDH and is highly expensive^[8].

Complete Blood Count (CBC) is the most commonly done laboratory test asked for almost all the febrile illnesses that might include malaria patients. It is available extensively in developing as well as developed countries. Even though the first automated analyser was built in 1953, it was not until 1990s when alterations in CBC was first noted and reported^[6,15]. Ever since, many studies have been conducted in malaria diagnosis with automation using Cell-Dyn (3500 and 4000), Coulter (GEN.S and LH) and Sysmex analysers. Results varied greatly in sensitivity and specificity from 48 to 100%, best being with studies involving Cell Dyn 4000 series and coulter^[5,6,8,9,16,17]. We evaluated the feasibility of LH750 Beckman Coulter as a rapid and cost effective method to detect Malaria especially in cases, which lacks clinical suspicion.

In our study, we concluded that SD in lymphocyte and monocyte volumes are most accurate in malaria detection, which was first reported in 2004 by Fourcade et al^[9]. He was the first to include the possibility of using a discriminant factor to raise a suspicion flag in the analyser. In his study, he used the same parameters and used a cut off value of 5.1 to obtain 96.9% sensitivity and 82.5% specificity. Subsequent study published in 2006 by Briggs et al^[8] had a better sensitivity and specificity (98% and 94%) using a different cut off value of

Comment [DBM9]: Reference number have been added. Similarly in 178 line reference number have been added to briggs et al.

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186 3.7. The malaria factor used for *P.falciparum*, *P.vivax* and *P.ovale* was 6.2, 5.9 and 5.7
187 respectively. Both studies showing malaria factor is not a fixed number and has to be
188 standardized to the machine and to the population who is being evaluated with that. We
189 analysed three groups with malaria positive, malaria negative with fever and healthy
190 individuals. By using logistic regression the malaria factor derived was using SD volume of
191 monocytes and lymphocytes. Cut-off of 2.7 as Malaria factor yielded specificity of 92.5%
192 and sensitivity of 83.7%.

193 The specificity and sensitivity obtained in findings from the present study is in
194 concordance with most of the previous studies except two studies^[5,6] that
195 published by Grobuschet al who got a which reported low sensitivity and Campuzano et
196 al who got variable results. Combining Malaria factor with other complimentary parameters
197 like variation in monocyte volume and platelet count reduction could increase the detection
198 rate, which needs further studies. Table 6 shows studies with their sensitivity and specificity
199 from literature^[18-22] regarding the use of Automated Haematology Instruments in Malaria
200 detection.

201 The present study demonstrated a fully automated rapid method for the potential detection
202 of malaria infection in adults. However, as already seen the malaria factor is not a constant
203 factor and needs to be standardized for the population. Advantage of this being results of the
204 malaria factor will be available at the same time as part of the CBC count and at no extra cost
205 because these data are reported automatically with the differential count results. Hence, this
206 can be implied as an effective method to raise a malaria suspect flag especially among the
207 malaria endemic area after standardization.

208 **Conclusion:**

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209 By using a previously described algorithm, a “suspect malaria” flag could be generated on
210 the analyser, allowing the detection of cases of unsuspected malaria and therefore, early
211 diagnosis of the disease with the potential of reducing the possibility of serious
212 complications.

Comment [DBM12]: I have deleted the part saying reduction in potential serious complication which needs to be proven as pointed out.

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213 We cannot consider the suspect malaria flag as gold standard diagnostic method. It should
214 be used as an indicator to screen the samples for malaria by specific methods when reported
215 the flag. Most of the studies on this modality of malaria detection being done in non-endemic
216 area and India being an endemic area demands more study towards automated detection of

217 Malaria. This study involves a small sample size from a specific area and hence, the positive
218 findings in the specific population cannot be extrapolated.

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Comment [DBM13]: Necessary changes in the conclusion made as advised.

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219 Limitations:

220 The present study involves a small sample size. Further studies with large sample size
221 and comparison with RPA technique may provide aid in development of a fully automated,
222 rapid, more sensitive and specific method for malaria detection. Standard values in mMalaria
223 factor are still unknown and varies with study and population hence needs to be
224 standardized for the population. Further prospective study is required to assess the
225 detection rate with the described malaria factor to establish its validity. Confounding factors
226 like age and gender is not being addressed in the study which if included as variables can
227 yield a more specific factor with better detection rate.

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Comment [DBM14]: Changes have been made including RPA technique comparison have been incorporated into it.

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Comment [DBM15]: Limitation have been added as suggested

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292 Table 1. Range of SD Volume of lymphocyte and monocyte

293 Table 2. Kruskalwallis test result for SD volume scatter and conductivity

294 Table 3. Mann Whitney U test comparing malaria positive, negative and control group.

295 Table 4. ROC analysis comparison among volume, conductivity and scatter

296 Table 5. Comparison of True positive, True Negative, False positive, False Negative,
297 PPV and NPV of the study

298 Table 6. Summary of studies evaluating Malaria diagnosis accuracy with automation

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Figure 1. Comparison of ROC of Malaria Factor calculated from SD Volume, SD Conductivity and SD Scatter ROC

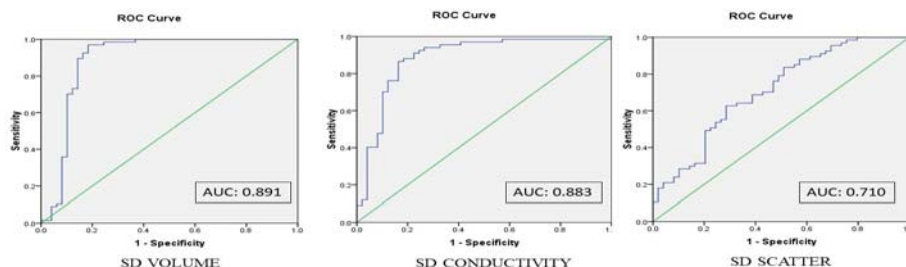


Figure 1: Comparison of ROC curve of Malaria factor calculated from the SD volume, SD Conductivity and SD Scatter.

SD = Standard Deviation; ROC= Receiver Operator Curve; AUC= Area Under Curve

Table 1: Range of SD Volume of lymphocyte and monocyte

Volume	Positive	Negative	Control
Lymphocyte SD	9.32 - 32.94	10.32 – 24.79	11.06 – 15.85
Monocyte SD	18.99 - 42.51	16.86 – 41.57	14.84 – 23.6

SD=Standard Deviation

Table 2: Kruskal wallis test result for SD volume scatter and conductivity

Variable		p Value
Volume	Neutrophils	.000
	Lymphocytes	.000
	Monocytes	.000
	Eosinophils	.111
Conductivity	Neutrophils	.000
	Lymphocytes	.000

Scatter	Monocytes	.000
	Eosinophils	.001
	Neutrophils	.000
	Lymphocytes	.000
	Monocytes	.103
	Eosinophils	.076

SD= Standard Deviation

The Kruskal Wallis test result for the SD volume scatter and conductivity

Table 3: Mann Whitney U test comparing malaria positive, negative and control group

Variable		Positive & Negative	Positive & Control	Negative & Control
Volume	Neutrophil	.183	.000	.000
	Lymphocyte	.000	.000	.048
	Monocyte	.000	.000	.000
	Eosinophil	.002	.130	.014
Conductivity	Neutrophil	.001	.203	.009
	Lymphocyte	.793	.000	.000
	Monocyte	.700	.000	.000
	Eosinophil	.573	.000	.000
Scatter	Neutrophil	.000	.000	.856
	Lymphocyte	.708	.000	.000
	Monocyte	.189	.068	.002
	Eosinophil	.029	.004	.491

Lymphocyte and Monocyte Standard deviation of volume were significantly different among all three groups.

Table 4: ROC analysis comparison among volume, conductivity and scatter

	Volume	Conductivity	Scatter
Cutoff value	2.7	0.4	0.54
Area under curve	0.891	0.883	0.710
Specificity	92.5%	91.0%	68.7%
Sensitivity	83.7%	75.5%	57.1%

ROC=Receiver Operator Curve

Table 5: Comparison of True positive, True Negative, False positive, False Negative, PPV and NPV of the study

	Volume	Conductivity	Scatter
True-positive	62	61	46
True-negative	41	37	28
False-positive	8	12	21
False-negative	5	6	21
PPV	88.6%	83.6%	68.7%
NPV	89.1%	86%	57.1%

PPV=Positive Predictive Value; NPV= Negative Predictive Value

Table 6: Summary of studies evaluating Malaria diagnosis accuracy with automation

Author	year	Instrument/ Test	Sensitivity (%)	Specificity (%)	Malaria factor
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Mendelow et al¹⁸	1999	Depolarized Laser Light	72	96	
Hanscheid et al²²	2001	CD3500, Abbott	95	88	
Grobusch et al⁵	2003	CD3000, Abbott	48.6	96.2	
Scott et al¹⁹	2003	CD4000, Abbott	80.2	87.3	
Yoo HJ et al²⁰	2010	Sysmex XE-2100	46.2	99.7	
Fourcade et al⁹	2004	Beckman Coulter	96.8	82.5	5.1
Briggs et al⁸	2006	Beckman Coulter	98	94	3.7
Kang et al¹⁶	2008	Beckman Coulter	81.8	72.3	4.57
Indira et al²¹	2015	Beckman Coulter	97	89	3.4
Present Study	2011	Beckman Coulter	92.5	83.7	2.7