

1

1 Original Article

2 **Comparative MALARIA DIAGNOSIS BY using LH750 Beckman Coulter** 3 **AUTOMATED**

4 **ANALYSER in India: A CROSS SECTIONAL CASE** 5 **CONTROL STUDY**

5 Running Title: Malaria Diagnosis using VCS data by Automation

6

7

8 **Abstract**

9 **Objective:** To study the VCS pattern in patients with malaria and compare it with patients
10 with fever who were malaria parasite negative and normal individuals and to determine
11 whether a further adaptation of the discriminant factor is needed using additional parameters
12 from the VCS data analysis using Beckmann Coulter LH 750.

13 **Design:** Cross sectional Case control study

14 **Setting:** Clinical Laboratory and Haematology Department, KMC, India

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

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

21 **Main Outcome measure:** Discriminant Factor

22 **Result(s):** We used the significant difference in the SD volume of the lymphocyte and
23 monocyte to differentiate the malaria positive from malaria negative. By using logistic

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25 regression, the “Malaria factor” is defined. By using a cut-off value of 2.7, specificity 24 of 92.5
26 and sensitivity of 83.7 were obtained.

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

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

32 **Introduction**

33 Malaria is a major health problem with over 40% of world’s population exposed to
34 varying degree of malaria risk. India is endemic to malaria with a case load of 1.49million in
35 2010^[1]. WHO estimates 216million cases with 445000 deaths worldwide in 2006^[2]

36 .

37 Cornerstones in global malaria control strategy is effective disease management by prompt
38 and accurate diagnosis. Earliest symptoms of malaria are nonspecific and hence the clinical
39 diagnosis is challenging and not reliable as it overlaps with most of the viral diseases and
40 febrile illnesses^[3,4]³⁸ .

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

Comment [T1]: This title can be better defined if all these parameters are added rather than generalized statement

Comment [T2]: What malaria parasite was investigated or evaluated e.g. P. Falciparum, Vivax?

Comment [T3]: Was there any ethical approval for this study and the consents of the patients sought?

Comment [T4]: Result section is about data in figures, values or percentages. There is need to robust this section with the available data from this study

Comment [T5]: Have you compared your outcome with RPA technique for Malaria parasites diagnosis?

have been reported as one of the main reasons for misdiagnosis^[5]41 . Parasitological diagnosis
42 of malaria is by Giemsa-stained Thick blood Film (GTF) that help in speciation and
43 diagnosis. Other methods like Quantitative Buffy Coat (QBC), Rapid Diagnostic Tests
44 (RDT), and PCR are not highly sensitive and thus not reliable in detection in low-density
45 malaria infections. Hence, the need for a sensitive and reliable test using laboratory
46 technology.

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Automated analysers uses VCS technology to quantify WBCs, it can 47 also detect the
48 infected hemozoin ingested WBC by the difference in volume conductivity and scatter as this
49 has a different scatter plot and this can be used as a screening method especially in endemic
area^[6,7]50 . Malaria detection in previous studies by automation used a discriminant factor
51 derived from differences in standard deviation (SD) of volume of lymphocytes and
monocytes^[8,9]52 . Factor was different in each study and varied with each species of
53 plasmodium. We analysed the VCS pattern in patients with malaria and compared with
54 malaria parasite negative fever patients and normal population to deduce a Malaria factor that
55 can be used in Indian population to detect the possibility of infection. This study gains
56 importance by being conducted in South Karnataka, which is Malaria endemic.

57 Objectives:

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

64 Methods

65 The study area (Udupi District) is endemic for malaria infection with 1188 cases reported
66 in 2010, of which 194 were *P. falciparum* infection. Our study was a Cross-sectional Case
67 control study with the blood samples collected from patients attending Hospital with fever or
68 clinically suspected malaria during the study period 2 months from July 2011 to August

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2011. The Ethical Committee of Institute approved study. Samples collected 69 were from adult
70 individuals were grouped into three groups.

71 □ Group 1: Patient with fever and diagnosed as Malarial Parasite positive by
72 fluorescent method (Quantitative buffy coat) and/or Immunochromatography.

73 Ninety-three patient sample during the study period formed the study group.

74 □ Group 2: Fifty samples of patient with fever and diagnosed as Malarial Parasite
75 negative by fluorescent method (Quantitative buffy coat) and/or
76 Immunochromatography.

77 □ Group 3: Hundred samples from apparently healthy adults in whom all CBC
78 parameters were within reference range and from voluntary blood donors formed
79 the Control group.

80 □ Patients with previously diagnosed systemic diseases presenting with fever were
81 not included in the study. We ensured precision of VCS data since we run two
82 level controls for CBC & differential count twice daily on Beckman Coulter LH

Comment [T6]: Crosscheck your findings with Recombinase Polymerase Amplification (RPA for Malaria diagnosis capable of detecting the pathogens at lower titre.

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Comment [T7]: This statement supports that the initial title of this study should be revisited and reframed.

Comment [T8]: What about the Ethical approval number given by the IRB/

Comment [T9]: How did you arrive at your sample size calculation?

83 750.

84 The quantitative buffy coat method (QBC) combines an acridine orange coated capillary
85 tube, an internal float, and separates layers of Platelets, WBCs and RBCs using
86 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]87 .

88 *Protocol*

89 We collected all peripheral blood samples in K3EDTA tubes and analysed within 2 hours
90 using LH750 Beckman Coulter for VCS data. We recorded the VCS data of the neutrophils,
91 lymphocytes, monocytes and eosinophils and compared between Malaria positive, Malaria

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negative and Control Group. In Malaria positive samples, there was volume 92 heterogeneity in
93 lymphocytes and monocytes.

94 *Variables*

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

97 *Study size*

98 All malaria positive patient samples during the study period July 2011 to August 2011
99 (2months), which was 93 cases comprised the study group. Fifty Malaria negative samples
100 confirmed by QBC/Immunochromatography and hundred samples from apparently healthy
101 individuals formed the control group.

102 *Statistical Analysis*

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

107

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

109

110 Receiver operating characteristic (ROC) analysis was performed on the malaria factor to
111 determine whether a satisfactory cut-off value could be established for the detection of
112 malaria in a blood sample. The results of the SD volume, conductivity & scatter of the

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neutrophils, lymphocytes, monocytes & eosinophils were analysed by 113 using the Kruskal
114 Wallis test to determine any statistical differences between three groups.

115 **Results**

116 During the 2 months study period, we found 93 cases to be malaria positive among the
117 suspected cases. All malaria positive cases out of the total 243 samples, had volume
118 heterogeneity in lymphocytes and monocytes as compared to malaria negative cases and
119 normal samples. Table 1 shows the range of Standard deviation (SD) of lymphocyte and
120 monocyte observed in the study. The difference in SD for the variables among all 3 groups
121 were statistically significant for SD volume of neutrophil, lymphocytes, monocytes, SD
122 conductivity of neutrophil, lymphocyte, monocyte, eosinophil, SD scatter of neutrophil and
123 lymphocyte as shown in Table 2.

124 On comparing the significant difference in mean volume, conductivity and scatter
125 between malaria positive, malaria negative and control groups, SD volume of Monocytes and

Comment [T10]: Did you evaluate these immunological parameters with direct detection of the pathogens via Microscopy and RDT to ascertain their detection correlation with the automated analyzer used and with what proof?

Comment [T11]: Please indicate the p value for this significance here

126 Lymphocytes among malaria positive cases were significantly different from negative and
127 control group (Table 3). Malaria factor was calculated using logistic regression. We did ROC
128 analysis for the malaria factor thus calculated from the equation using SD of Volume,
129 Conductivity and Scatter. Figures 1 shows the ROC curve of Malaria factor calculated from
130 the SD volume, Conductivity and Scatter. Table 4 summarizes the findings of the ROC
131 analysis between volume, conductivity and scatter.
132 True positive, true negative, false positive and false-negative results obtained in Volume,
133 Conductivity and Scatter are given in Table 5. These figures were used to calculate the
134 positive predictive value (PPV) and negative predictive value (NPV) for this study. From the
135 findings, the Malaria Factor obtained using the SD volume difference in lymphocyte and

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monocyte has the highest specificity and sensitivity of 92.5 and 83.7 respectively 136 with a PPV
137 of 88.6% and NPV of 89.1%.

138 Discussion

139 Malaria is not only a health issue for the developing country but an economic burden. It is
140 one of the most common parasitic diseases in the world [Ref](#). Morbidity of the disease is
attributed

141 to delay in diagnosis, the mainstay of which is clinical suspicion. The “gold standard”
142 diagnostic modality for malaria is still Giemsa-stained Thick blood Film (GTF), microscopic
143 examination of stained thick and thin blood films. Disadvantage being it is difficult,
timeconsuming

and low levels of parasitemia may be missed^[11]144 .

QBC technique is used as a backup to blood films and to simplify malaria diagnosis^[12]145 .

The overall sensitivity rate for QBC in a study by Adeoye et al^[13] 146 was 55.9% when compared
147 with GTF. Sensitivity of QBC and GTF reduces further with samples having low parasite
148 density. QBC test cannot differentiate accurately between different Plasmodium species but
149 with the GTF. In spite of the speed and simplicity of QBC technique, it cannot be considered
150 an acceptable alternative to GTF under routine clinical laboratory situation. The
151 disadvantages of this method are the high cost of the equipment, nonspecific nature of stain
152 and consumables. Many rapid methods are present in the market with variable detection rate,
153 which still **needs need** clinical suspicion to be asked for. High false positivity in high WBC
counts

154 or in leukemic patients, persisting histidine rich proteins for days after treatment, inability to
155 detect *P. ovale* and *malariae*, prozone effect and inability to quantify adds as the disadvantage
for these methods^[14]156 . Polymerase chain reaction has been proven to be sensitive in the
157 diagnosis of all four species of malaria. However, it is expensive and impractical in the
158 routine diagnosis of malaria.

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Sensitivities of immunologic methods for detecting malaria remain 159 a problem. In non160
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]161 .

162 Complete Blood Count (CBC) is the most commonly done laboratory test **asked**
requested/demanded for

163 almost all the febrile illnesses that **might may** include malaria patients [Refs](#). It is available
extensively

Comment [T12]: You have enough data in your result to enrich the result section of your abstract. I suggest you revisit your abstract as earlier pointed out.

Comment [T13]: Did you compare and evaluate your findings with this established statements?

Comment [T14]: Meaning of Acronym

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164 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]165 .
166 Ever since, many studies have been conducted in malaria diagnosis with automation using
167 Cell-Dyn (3500 and 4000), Coulter (GEN.S and LH) and Sysmex analysers. Results varied
168 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]169 . We evaluated the feasibility of LH750 Beckman
170 Coulter as a rapid and cost effective method to detect Malaria especially in cases, which

lacks

171 clinical suspicion.

172 In our study, we concluded that SD in lymphocyte and monocyte volumes are most

173 accurate in malaria detection, which was first reported in 2004 by Fourcade et al.^{Ref Number.}

He was the

174 first to include the possibility of using a discriminant factor to raise a suspicion flag in the
175 analyser. In his study, he used the same parameters and used a cut off value of 5.1 to obtain
176 96.9% sensitivity and 82.5% specificity. Subsequent study published in 2006 by Briggs et al
177 had a better sensitivity and specificity (98% and 94%) using a different cut off value of 3.7.
178 The malaria factor used for *P.falciparum*, *P.vivax* and *P.ovale* was 6.2, 5.9 and 5.7
179 respectively. Both studies showing malaria factor is not a fixed number and has to be
180 standardized to the machine and to the population who is being evaluated with that. We
181 analysed three groups with malaria positive, malaria negative with fever and healthy
182 individuals. By using logistic regression the malaria factor derived was using SD volume of

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monocytes and lymphocytes. Cut-off of 2.7 as Malaria factor yielded specificity 183 of 92.5%
184 and sensitivity of 83.7%.

185 The specificity and sensitivity obtained in the present study is in concordance with most
186 of the previous studies except that published by Grobuschet all who got a low sensitivity and
187 Campuzano et al who got variable results. Combining Malaria factor with other
188 complimentary parameters like variation in monocyte volume and platelet count reduction
189 could increase the detection rate, which needs further studies. Table 6 shows studies with
their sensitivity and specificity from literature^[18–22] 190 regarding the use of Automated
191 Haematology Instruments in Malaria detection.

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

199 **Conclusion:**

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

204 We cannot consider the suspect malaria flag as gold standard diagnostic method. It should
205 be used as an indicator to screen the samples for malaria by specific methods when reported

Comment [T15]: Same as above in referencing

Comment [T16]: Recast this statement

Comment [T17]: This statement needs to be substantiated and scientifically proven

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the flag. Most of the studies on this modality of malaria detection being done 206 in non-endemic 207 area and India being an endemic area demands more study towards automated detection of 208 Malaria.

209 **Limitations:**

210 [The present study involves a small sample size. Further studies with large sample size 211 may provide fully automated, rapid, more sensitive and specific method for malaria detection. 212 Malaria factor varies with study and population hence needs to be standardized for the 213 population. Further prospective study is required to assess the detection rate with the 214 described malaria factor to establish its validity. Confounding like age and gender is not 215 being addressed in the study which if included as variables can yield a more specific factor 216 with better detection rate.

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Comment [T18]: There is also the need for re-evaluation with the gold standard including PCR

Comment [T19]: Poor referencing

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279

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

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

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

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

284 Table 5. Comparison of True positive, True Negative, False positive, False Negative,
285 PPV and NPV of the study

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

287

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

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Table 1: Range of SD Volume of lymphocyte 291 and monocyte

292

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

293 SD=Standard Deviation

294

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

296

Variable p Value

Volume Neutrophils .000

Lymphocytes .000

Monocytes .000

Eosinophils .111

Conductivity Neutrophils .000

Lymphocytes .000

Monocytes .000

Eosinophils .001

Scatter Neutrophils .000

Lymphocytes .000

Monocytes .103

Eosinophils .076

297

298 SD= Standard Deviation

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

300

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Table 3: Mann Whitney U test comparing malaria positive, negative 302 and control group

303

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

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

306 **Table 4: ROC analysis comparison among volume, conductivity and scatter**
307

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%

308 ROC=Receiver Operator Curve

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**Table 5: Comparison of True positive, True Negative, False positive, False 310 Negative, PPV
311 and NPV of the study**

312

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%

313

314 PPV=Positive Predictive Value; NPV= Negative Predictive Value

315

316

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

318

Author year

Instrument/

Test

Sensitivity

(%)

Specificity

(%)

Malaria

factor

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

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16

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

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