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Evaluation of Sensitivity and Specificity of High Fluorescence Lymphocyte Count Percentage of Sysmex XN Analyzer in Diagnosis of Dengue

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Abstract:

BACKGROUND: The Sysmex XN-series hematology analyzers provide newer parameters including high fluorescence lymphocyte cell percentage (HFLC%) which correlates with the presence of atypical lymphocytes in peripheral blood. We aimed to analyze the sensitivity and specificity of HFLC% as a diagnostic tool and its association with serological status in diagnosed dengue patients and thereby establish a cutoff of HFLC% based on serology. Besides, we also wish to correlate HFLC% with thrombocytopenia in these patients.

MATERIALS AND METHODS: A total of 1500 serum samples were subjected to serological evaluation for dengue. After excluding hematological malignancies and autoimmune disorders, the same day complete blood count parameters including HFLC% and platelet counts were collected retrospectively for 292 serologically positive dengue cases and 76 seronegative controls.

RESULTS: Our result shows that in nonstructural 1 antigen-positive cases, a cutoff of >5.2% HFLC can have a sensitivity of 79.5% and specificity of 98.6%. We found a different cut off of HFLC% >3.2% (sensitivity 83.4%, specificity 98.6%) for the cases with only immunoglobulin M positivity and a cut off of HFLC% >2.6% (sensitivity 86.1%, specificity 96%) in the dual positive cases (immunoglobulin M with nonstructural 1 antigen). Besides, high HFLC% also shows a strong correlation with platelet count with a Spearman correlation coefficient of −0.6.

CONCLUSIONS: The result of our study shows that a specific cutoff of HFLC% can not only help us to suspect dengue fever but also predict the risk of thrombocytopenia in already diagnosed dengue patients. The sensitivity and specificity of HFLC% varied with the serological status of the patients which depend on the days of fever on presentation.

Keywords:

Dengue, high fluorescence lymphocyte cell, platelet, reactive lymphocyte, Sysmex

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Introduction

Sysmex XN-series automated hematology analyzer (AHA) provides a five-part differential with cluster resolution and a clear separation of some of the abnormal cells circulating in peripheral blood. This AHA differentiates white blood cells by means of their size, internal complexity, and overall nucleic acid content. On the basis of three parameters, forward scatter, side scatter (SSC), and side fluorescence (SFL), these machines generate scattergrams and cell population data, helping in suspicion of various disease conditions. Besides, XN-1000 AHA also has an automated flagging system to suspect atypical lymphocytes.

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The high fluorescence lymphocyte cell percentage (HFLC%) and count are research parameters available in the Sysmex AHA.[1] These are observed in the white blood cell differential (WDF) channel as an area of high fluorescence above the lymphocyte and monocyte area. The characteristic high fluorescence intensity is because of high ribonucleic acid content in these cells. HFLC has been studied in the identification and quantification of antibody secreting cells (activated B lymphocytes) and found to have high precision and reliability in patients without systemic hematological diseases.[2,3] Its sensitivity and specificity in the detection of peripheral plasma cells have also been studied.[4] HFLC was further studied to differentiate between common causes of febrile illnesses with thrombocytopenia in dengue-endemic areas.[5] All of these studies were based on different series of Sysmex analyzers.

Various studies have reported the association of platelet with the serological profiles of dengue infection.[6,7] The reason for thrombocytopenia in dengue is varied. Some of the hypotheses are being bone marrow suppression and the peripheral destruction of platelets by antiplatelet antibodies.[8]

In our study, we wish to observe the association of high HFLC% in XN-1000 series analyzer, with serologically diagnosed cases of dengue, its relation with either and both of nonstructural 1 antigen (NS1Ag) and dengue immunoglobulin M (IgM) antibody-positive status and whether there is any correlation of HFLC% with the same day platelet count. We also wish to establish a cutoff of HFLC% based on their serological status.

Materials and Methods

Study design

This is a retrospective study.

Sample size

One thousand five hundred peripheral blood samples in ethylenediaminetetraacetic acid (EDTA) vacutainers (Becton Dickinson Vacutainers Systems Europe, Meylan, France) already subjected to serological evaluation for dengue were recruited for screening. Complete hemogram report by Sysmex XN-series AHA on the day of serological testing was collected to evaluate HFLC% for total 292 serologically positive dengue cases and 76 nondengue age and sex-matched controls. Patients with systemic hematological disorders (such as leukemia or lymphoma) and autoimmune disorders were excluded from study. Control population was defined by the presence of fever as well as dengue seronegative status. We exclusively included the same day HFLC% and platelet count for evaluation. As shown in Figure 1, dengue cases were further divided into NS1Ag, IgM antibody, and dual-positive subgroups with 93, 163, and 36 cases, respectively, and compared with controls separately. Dengue NS1Ag was detected by Panbio Dengue Early enzyme-linked immunosorbent assay (ELISA) kits (Abbott, Chicago, IL, USA). Dengue IgM was tested by Dengue IgM capture ELISA (National Institute of Virology, Pune, India).

Analyzer principle

All the samples were processed in the Sysmex XN-1000, which incorporates the Sysmex XN-10 AHA module with a sampler. The AHA was run in accordance with the standard operating procedure and was checked daily with internal quality checks of low, normal, and high levels. Calibration of the analyzer was done once a year. All the samples were run within 4 h of collection in dipotassium EDTA tubes. It analyzes cells based on impedance and flow-cytometric characteristics, using semiconductor laser and fluorescent dye. Cells with higher SFL falling above the lymphocyte population are gated by the AHA as HFLC. Figure 2 provides two representative scattergrams of WDF (SSC-SFL) channel; Figure 2a shows a normal scattergram and Figure 2b shows HFLC population (as encircled).

We studied platelet-related parameters along with HFLC% for all recruited patients.

Statistical analysis

Receiver operating characteristic (ROC) curve was generated, and Youden's index was calculated for a specific cutoff HFLC% to suspect dengue. The same analysis was done for platelet count, and a correlation between HFLC% and thrombocytopenia was also evaluated. Analysis was done using MedCalc statistical software version 19.4.1 (MedCalc Software Ltd, Ostend, Belgium) and GraphPad prism version 8.0.0 for Windows, GraphPad software, San Diego, CA, USA.

Ethical clearance

Ethical clearance was taken from Institute Ethical Committee of All India Institute of Medical Sciences, New Delhi.

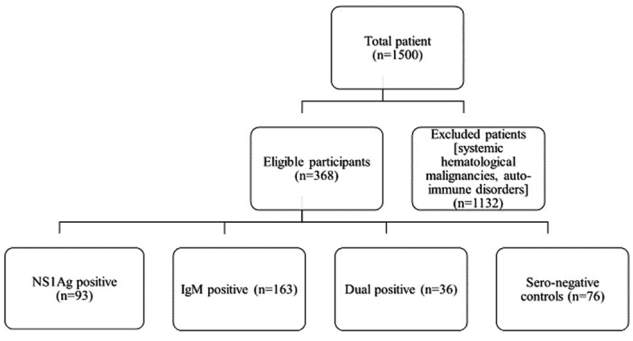

Figure 1: Flow diagram of patient recruitment into different subgroups

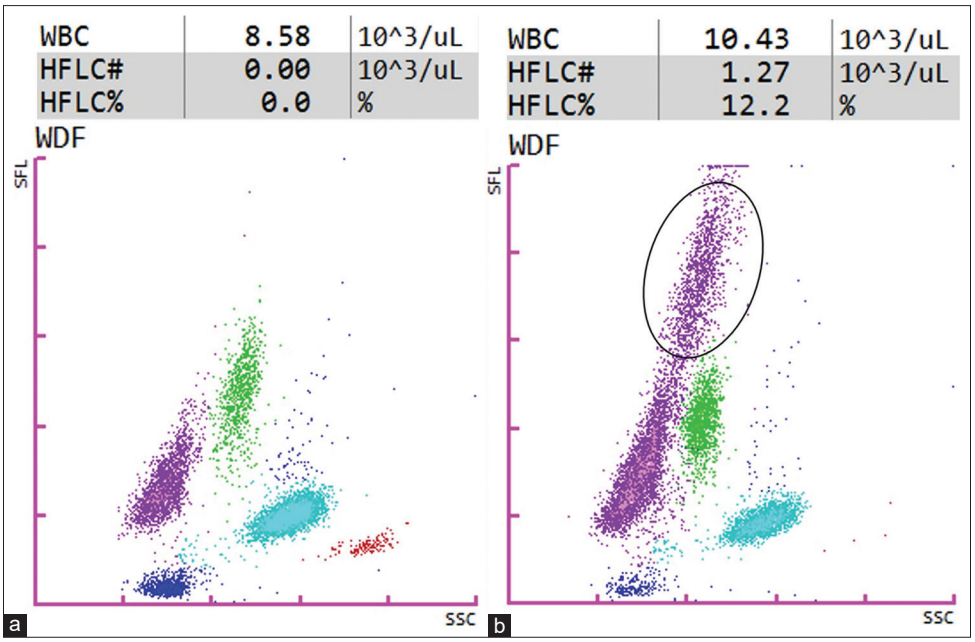

Figure 2: Comparison of a normal white cell differential (side scatter-side fluorescence) scattergram with that of a case with high fluorescence lymphocyte cell percentage. (a) shows a normal scattergram while (b) shows scattergram with high fluorescence lymphocyte cell percentage

Results

Our study design included four subgroups, namely seronegative control, NS1Ag positive, IgM antibody positive, and dual-positive cases. All compared variables were in nonnormal distribution. As shown in supplementary Figure 1, age distribution among four subgroups showed no significant variation in age distribution ($P = 0.0831$) by Kruskal–Wallis test. Table 1 summarizes the median and interquartile range of variables in different subgroups.

Figure 3a depicts HFLC% distribution among four subgroups, observing significant variation in HFLC% value distribution in dengue seropositive patients from seronegative controls ($P < 0.0001$) by Kruskal–Wallis test. One-way ANOVA was applied and a significant strength of association with a value of 0.36 was found.

We also compared the intergroup difference of HFLC%. Tukey's multiple comparison analysis showed a significant difference between seropositive and seronegative subgroups, but the difference was not significant among the seropositive subgroups.

All three seropositive subgroups were compared separately with seronegative controls to generate a ROC curve and cutoff percentage for HFLC% by Youden's index analysis. Table 2 summarizes sensitivity and specificity of a particular HFLC% cutoff for suspecting seropositive dengue patients.

Figure 3b-d outline the ROC curves for HFLC% in NS1Ag positivity, IgM positive and dual positive cases respectively. We observed for NS1Ag positivity, a >5.2% HFLC cut off can have 79.5% sensitivity and 98.6% specificity. Youden's index analysis showed in IgM positive cases, an HFLC cutoff of >3.2% has a sensitivity of 83.4% and specificity of 98.6%. For dual positive cases, we found a >2.6% HFLC value has a sensitivity of 86.1% and specificity of 96%.

We also analyzed the platelet count in different subgroups. As depicted in Figure 4a, the medians of platelet count vary significantly among dengue-negative and seropositive cases by Kruskal–Wallis test. One-way ANOVA was applied and we observed, though the variation in platelets among different subgroups is significantly associated with seropositive dengue, the strength of association is 0.11.

We also compared intergroup differences of platelet count Tukey's multiple comparison analysis showed a significant difference between seropositive and seronegative subgroups, but the difference was not significant among the seropositive subgroups. Figure 4a depicts the comparison in platelet count distribution among different subgroups.

Figure 4b-d shows the ROC curve for platelet count associated with NS1Ag, IgM, and dual positivity, respectively. Platelet cutoff was calculated by Youden's analysis. We found that platelet cutoffs for different subgroups are providing greater specificity for seropositive cases at the cost of

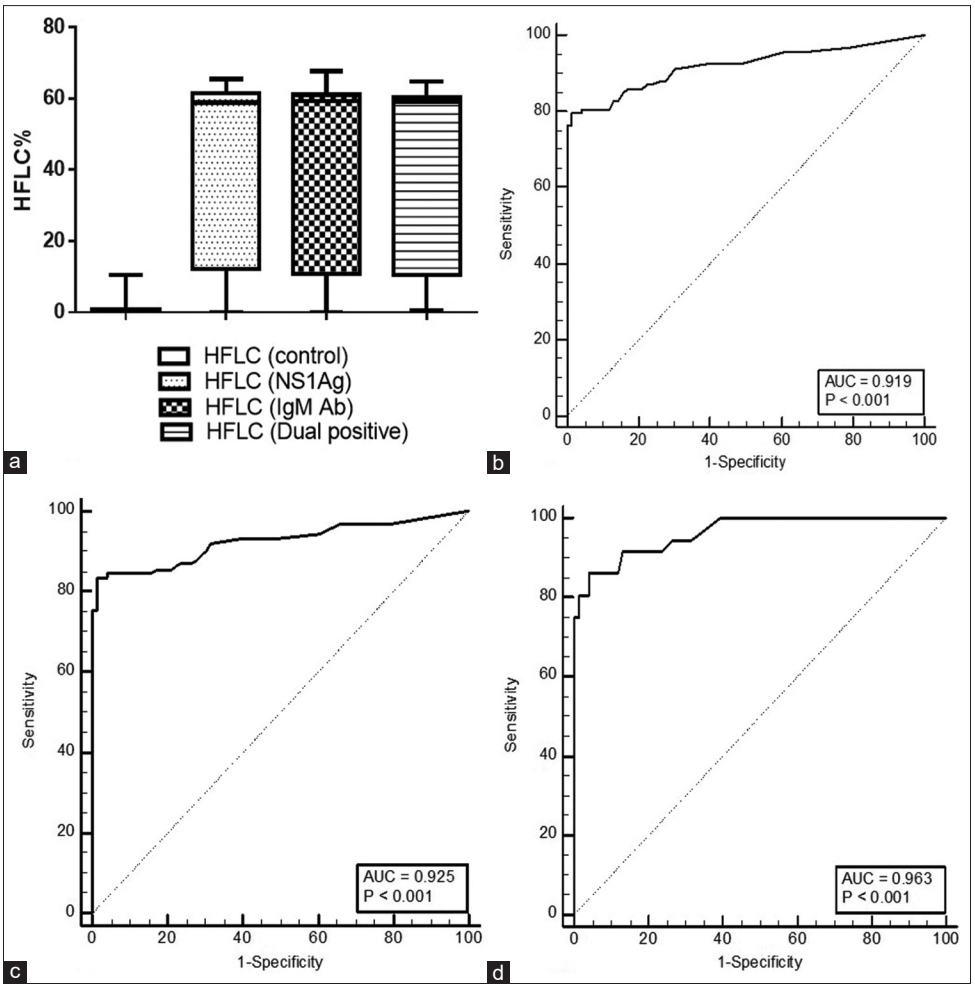

Figure 3: (a) shows variation of high fluorescence lymphocyte cell percentage distribution among different subgroups (b) depicts the receiver operating characteristic curve of high fluorescence lymphocyte cell percentage for dengue nonstructural 1 antigen positivity (c) shows the receiver operating characteristic curve of high fluorescence lymphocyte cell percentage for dengue immunoglobulin M positivity (d) illustrates the receiver operating characteristic curve of high fluorescence lymphocyte cell percentage for dual positivity

Table 1: The median and interquartile range of variables in different subgroups

Variables	Control	NS1Ag	IgM	Dual positive
Age (years)	26.5 (16-38)	25 (18.5-33)	24 (14-30)	26 (19.5-35)
HFLC%	0.76 (0.1-0.9)	43.7 (12.2-61.4)	43.3 (10.7-61)	42.1 (10.5-60.2)
Platelet ($\times 10^9$/L)	163 (53.5-287)	100 (46.5-186.5)	78 (36-142)	62.5 (31.2-112)

HFLC%=High fluorescence lymphocyte cell percentage; NS1Ag=Nonstructural 1 antigen; IgM=Immunoglobulin M

Table 2: Sensitivity and specificity of a specific high fluorescence lymphocyte cell percentage cutoff for suspecting seropositive dengue patients

Serology status	HFLC%	Sensitivity	Specificity	Area under the curve
NS1Ag	>5.2	79.5	98.6	0.919
IgM	>3.2	83.4	98.6	0.924
Dual positive	>2.6	86.1	96	0.963

HFLC%=High fluorescence lymphocyte cell percentage; NS1Ag=Nonstructural 1 antigen; IgM=Immunoglobulin M

sensitivity. A platelet cutoff <36 × 10^9/L showed 20.4% sensitivity (95% CI of 12.7%–30%) and 88.1% (95% CI of 78.7%–94.4%) specificity for NS1Ag-positive cases. The same for IgM and dual-positive cases was found to be <43 × 10^9/L (sensitivity 33.7% with 95% CI of 23%–37.7% and specificity 82.8% with 95% CI of 72.5%–90.5%) and <50 × 10^9/L (sensitivity 41.6% with 95% CI of 25.5%–59% and specificity 80.2% with 95% CI of 69.5%–88.5%), respectively. Supplementary Table 1 summarizes sensitivity and specificity of platelet cutoff values for suspecting seropositive dengue patients.

As we found, unlike thrombocytopenia, a high HFLC% is both specific and sensitive, we calculated odds ratio for each, which showed that HFLC% has a diagnostic odds ratio of 301, 378, and 150 for NS1Ag, IgM, and dual-positive cases, respectively, while thrombocytopenia has the same at 2, 2.4, and 2.9 for NS1Ag, IgM, and dual-positive cases, respectively.

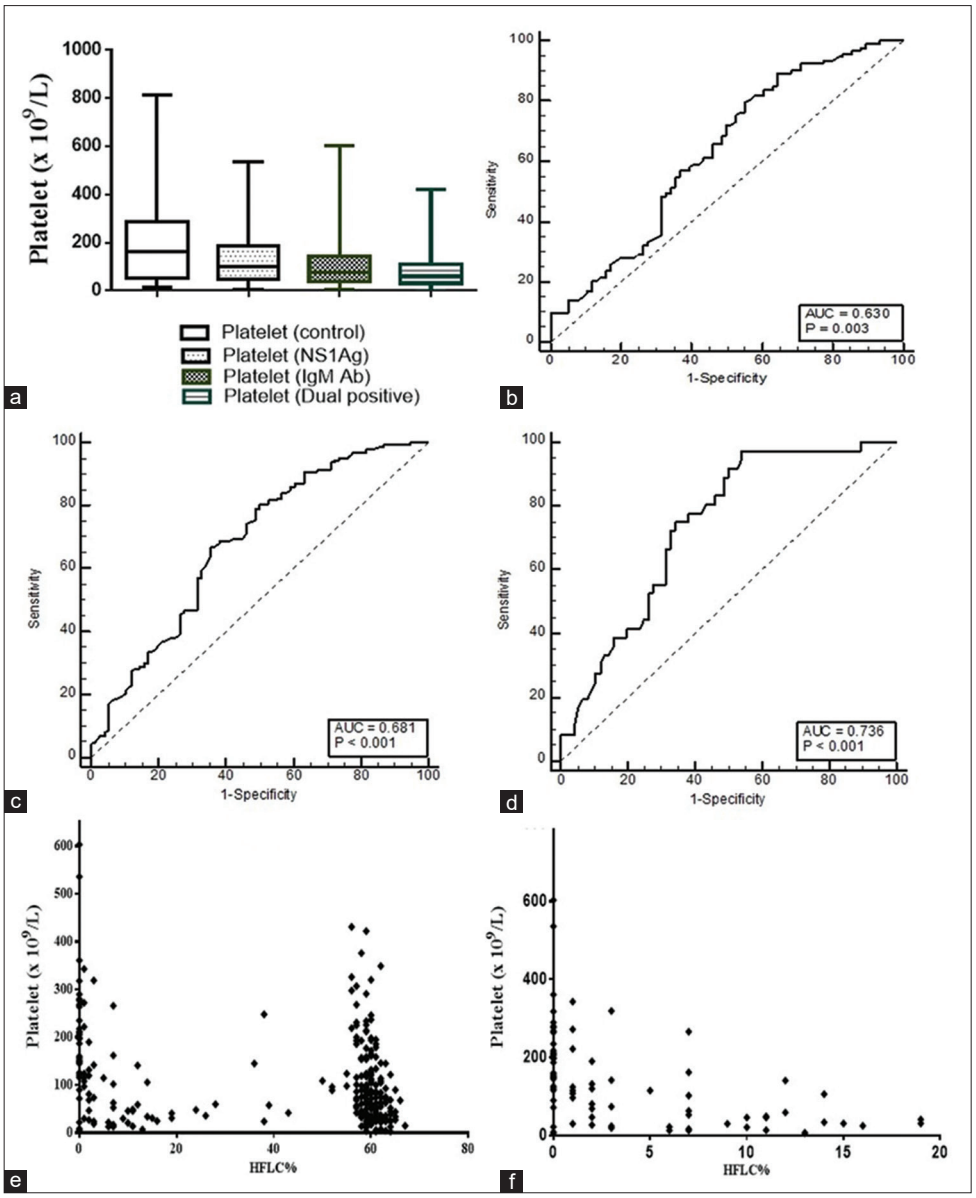

Figure 4: (a) shows variation of platelet distribution among different subgroups (b) depicts the receiver operating characteristic curve of platelet count for dengue nonstructural 1 antigen positivity (c) illustrates the receiver operating characteristic curve of platelet count for dengue immunoglobulin M positivity (d) shows the receiver operating characteristic curve of platelet count for dual positivity (e) shows correlation of platelet count with high fluorescence lymphocyte cell percentage (f) depicts correlation of platelet count with high fluorescence lymphocyte cell percentage (at values <20%)

Further analysis revealed that higher HFLC% was significantly correlated with platelet number with a Spearman correlation coefficient of −0.340. Figure 4e represents the correlation of HFLC% with platelet numbers. However, the platelet count versus HFLC% correlation plot had significantly more correlation when the HFLC% was under 20% showing a Spearman correlation coefficient −0.6. Figure 4f represents the correlation of HFLC% (<20%) with platelet count.

Discussion

Dengue, a viral hemorrhagic fever, is caused by the dengue virus (DENV). DENV belongs to the family *Flaviviridae* and genus *Flavivirus* and is transmitted to humans through the bite of female *Aedes* mosquito, mainly *Aedes aegypti*.[9] *Aedes albopictus* has been identified as a secondary vector of importance.[10] DENV has four serotypes, namely DENV-1, DENV-2, DENV-3, and DENV-4.[9]

Before 1970, only 9 countries had experienced severe dengue epidemics. The disease is now endemic in more than 100 countries in the World Health Organization (WHO) regions of Africa, the Americas, the Eastern Mediterranean, South-East Asia, and the Western Pacific. The America, South-East Asia, and Western Pacific regions are the most seriously affected, with Asia representing ~ 70% of the global burden of disease.[11]

It is a very common infection in tropical and subtropical climates, with an estimated 100-400 million infections globally per year. The largest number of dengue cases ever reported globally was in 2019.[11]

Infection with any of the DENV serotypes may be asymptomatic in the majority of cases or may result in a wide spectrum of clinical symptoms, ranging from a mild flu-like syndrome known as dengue fever to the most severe forms of the disease, which are characterized by coagulopathy, increased vascular fragility, and permeability as dengue hemorrhagic fever (DHF) or may progress to hypovolemic shock as dengue shock syndrome (DSS).[6-9]

The WHO classified dengue into dengue (with/without warning signs) and severe dengue. This subclassification is to help in triaging patients for hospital admission and close monitoring.[11] Severe dengue is commonly associated with a second infection by a different serotype, mostly because of incomplete neutralization of virus by an already existing antibody directed against the primary serotype. This phenomenon is known as "Original antigenic sin."[9]

The pathogenesis of dengue infection has various hypotheses, be it DENV tropism for peripheral blood mononuclear cells, cells of the reticuloendothelial system (RES) and endothelial cell, tropism for organs primarily liver and RES, the virulence of the virus itself, complement system activation, transient autoimmunity, host genetic factors, antibody-dependent enhancement, cross-reactive T-cell response, and soluble factors.[9]

Infected cells die through apoptosis and to a lesser extent through necrosis. Necrosis releases toxic products, which activate the coagulation and fibrinolytic systems. A high viral load in blood and viral tropism for endothelial cell, severe thrombocytopenia, and platelet dysfunction along with high concentration of cytokine results in increased capillary fragility, clinically manifested as petechiae, easy bruising, and gastrointestinal mucosal bleeding which is characteristic of DHF. At the same time, infection stimulates the development of specific antibody and cellular immune responses to DENV.[9]

Diagnosis of dengue can be by virological tests (directly detect elements of the virus, here NS1Ag) and serological tests, which detect human-derived immune components that are produced in response to the virus, here IgM. Depending on the time of patient presentation, the application of different diagnostic methods may be more or less appropriate.[11]

When using the entire data set, we found that age has no significant variation and concurred with the study by Dos Santos Carmo *et al*.[12]

Studies on different hematological and biochemistry profiles of dengue patients have been reported already.[12-14] A study evaluating HFLC as one of the parameters to differentiate between common causes of febrile illnesses with thrombocytopenia in dengue-endemic areas has been reported.[5] Similar to their study, we found the HFLC% in dengue patients to be significantly higher than control samples. The distribution of HFLC% among the dengue-positive subgroups was not found to be significantly different. This finding is in concordance with the study by Raharjo and Hadi.[15] In our study, we also evaluated the association of HFLC% with NS1Ag, dengue IgM, and dual-positive status.

Various studies associating dengue virological and serological findings with platelet count have been done.[6,7] Work on platelet activation determining the severity of thrombocytopenia in dengue infection is already reported.[16] Daily platelet count aiding in predicting DSS has also been reported.[17] In our study, we found that the medians of platelet count vary significantly among the dengue negative and positive cases.

Studies on HFLC parameters and its correlation with atypical lymphocytes and plasma cells are rare and few.[2-4] However, the available literature suggests a strong association between "atypical lymphocyte" flag in AHA and clinical severity of the disease. Besides, one study correlating serotype and hematological profile found that the percentage of atypical lymphocytes was associated with thrombocytopenia in DENV-1 and with severe leukopenia in DENV-3.[16] Similarly, another study correlating the atypical lymphocytes count with the severity of dengue infection has been reported.[18,19] As shown in Figure 4f, we found that a higher percentage of atypical lymphocytes, which are flagged by the Sysmex AHA as HFLC, is more associated with thrombocytopenia. This finding is in concordance with the study by Clarice *et al.* where they also found significant negative correlation of thrombocytopenia with HFLC%.[18] Further, on correlating HFLC% with platelet, we found that the platelet count versus HFLC% correlation plot has significantly more correlation when the HFLC% is under 20%. This can be reasoned that our study center, a tertiary care medical institute, receives undiagnosed as well as complicated and referred patients of dengue. Therefore, the patients with very high HFLC% (>20%) may already have received some form of treatment at other centers. Besides, different serotypes of viruses can have different associations with thrombocytopenia as already reported by other researchers.[16]

Our study has limitations that being a retrospective study, we could not evaluate the importance of HFLC

with the number of days of fever of the patients. However, highlighting the fact that NS1Ag and IgM testing is done when symptoms of dengue infection are <5 days and >5 days, respectively, the higher association of HFLC% with IgM indirectly suggests a higher antibody-mediated response with longer duration of fever.

Conclusion

We wish to conclude that the astute importance of atypical lymphocytes of dengue, flagged by the Sysmex AHA as HFLC%, has not only a fascinating association with dengue infection but also its value can have wide implications. The HFLC% should be monitored along with platelet count in any case of dengue infection. An increase of this parameter over specific cutoff as has been established in our study with >5.2% in NS1Ag-positive cases, >3.2% in IgM cases, and >2.6% in dual-positive cases will not only help us to diagnose but also to monitor the patient during the course of disease.

As dengue infection affects many tropical and subtropical countries, a parameter such as HFLC% which can be easily available through a simple complete blood count procedure will not only aid in diagnosis but also in management and monitoring.

Acknowledgments

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Nil.

Conflicts of interest

There are no conflicts of interest.

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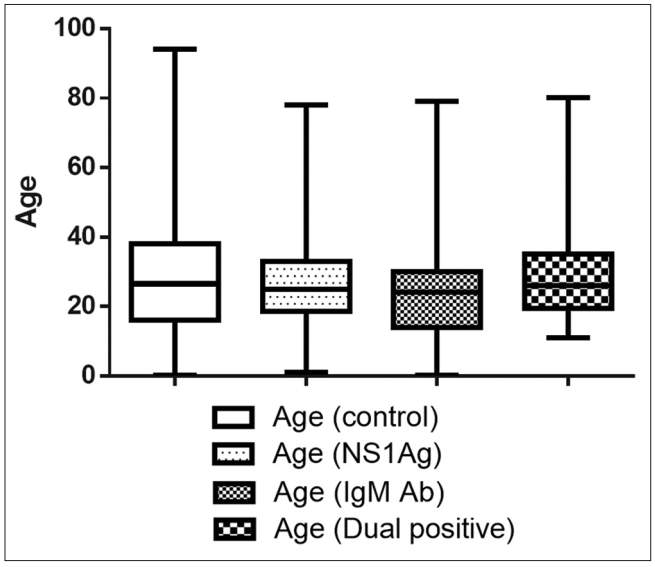
Supplementary



Supplementary Figure 1: Variation in age distribution among different subgroups

Supplementary Table 1: Sensitivity and specificity of platelet cutoff values for suspecting seropositive dengue patients

Serology status	Platelet count (10^9/L)	Sensitivity (%)	Specificity (%)	Area under the curve
NS1Ag	<36	20.4	88.1	0.630
IgM	<43	33.7	82.8	0.680
Dual positive	<50	41.6	80.2	0.735

NS1Ag=Nonstructural 1 antigen; IgM=Immunoglobulin M