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Utility of Atypical Lymphocytes and Large Immature Cells in Prediction of Dengue Severity

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Abstract: ***Background: There is a clinical imperative to devise metrics to prognosticate dengue severity. Our objective was to determine the association between longitudinal trends in atypical lymphocytes and large immature cell count with platelet count and dengue severity. Materials and methods: Retrospective analysis of longitudinally measured clinical and hematological data from (n = 79) hospitalized dengue patients was done. Results: The cohort consisted of patients with dengue fever without warning signs (DFWOWS) (n = 40, females = 14, and age = 19.9 ± 14.6 years), dengue fever with warning signs (DFWWS) (n = 36, females = 13, and age = 16.1 ± 14.1 years) and severe dengue (n = 3, females = 2, and age = 5.3 ± 4 years). Platelet count increased at a rate of 11, 524 cells/mm3/day, with a slower rate of rise as the severity increased (p = 0.001***). Concurrently hematocrit and neutrophil percentage decreased, while the lymphocyte percentage and white blood cell (WBC) count increased during the hospital stay. Every 1% increase in atypical lymphocyte count (ATY) was associated with a fall in platelet count by 16, 963 cells/mm3 (p = 0.001***). A similar but weaker trend was found for large immature cells (LICs). Conclusion: The data support the usefulness of longitudinal tracking of atypical lymphocyte and large immature cell count for dengue prognosis. The time trends of the hematological parameters indicate the progression of patients from the critical to the recovery phase.***

Keywords: Dengue, Atypical Lymphocytes

1. Introduction

Dengue is a mosquito - borne viral hemorrhagic fever caused by the Dengue virus, a flavivirus. It is transmitted by the bite of infected Aedes mosquitoes. This disease is principally seen in tropical and subtropical regions between the latitudes 3° N and 35° S.1 It is estimated that there are 390 million dengue virus infections globally every year, of which 96 million show clinical manifestations and approximately 40, 000 succumb to the disease annually.2 In India, 7, 95, 211 cases were reported, with 1, 151 fatalities for 2017–2022, with an average yearly of 1, 32, 535 cases and 192 deaths.3 Dengue virus comprises four predominant serotypes (DEN - 1, 2, 3, 4) with positive - sense single - stranded RNA as the genetic material and host membrane - derived lipid envelope. The virus is endowed with three structural proteins (capsid C, membrane M, and envelope E) and seven nonstructural (NS) proteins.1

Any of the four virus serotypes can cause illness, but most infections are asymptomatic. Primary infection induces lifelong immunity specific to the causal serotype. Second infection by a different serotype is thought to increase the risk for severe dengue. Antibodies induced by primary infection cross - react with the virus during the secondary infection in a nonneutralizing manner. Instead of clearing the virus, these antibodies facilitate the entry of the virus into cells that bear antibody receptors. The virus thrives inside the cell, creating an inflammatory cascade that culminates in the cardinal features of severe dengue, viz, vascular leakage, fluid accumulation, thrombocytopenia, shock, and potential death.

Vascular leakage due to endothelial damage causes a rise in hematocrit, and fluid accumulation in the lungs and peritoneum, with severe leakage resulting in hemodynamic collapse and multiple organ dysfunction. Shock can also progress to disseminated intravascular coagulation, which, in the background of thrombocytopenia, can cause life threatening hemorrhage. In addition to the antibody - medicated enhancement, cross reactive T cells, NS1 antigen, antidengue virus NS1 antibodies, and autoimmunity contribute to the pathophysiology of dengue complications.1, 4 The natural history of dengue fever has three stages/phases1, 5-(1) the acute febrile phase, (2) the critical phase following the defervescence, and (3) the recovery phase. Severe dengue with all attendant complications generally occurs after the febrile phase in the critical stage when the hematocrit rises and the platelets fall. After the peak/nadir, hematocrit and platelet return to the baseline values in the latter part of the critical phase as the patient progresses into recovery.

A clinical imperative is to devise metrics to predict the onset and extent of severe dengue and its underlying pathophysiology. Several markers have been explored for their prognostic purpose.4, 6–9 Atypical lymphocyte has been proposed as a helpful metric in dengue and other viral infections for assessment of severity and prognostication.10–12 In the present work, we focus on atypical lymphocytes and large immature cells (LICs), which are the research parameters returned by modern five - part colters. The present study is an exploratory study based on retrospective analysis of clinical and hematological data routinely collected in hospitalized dengue patients serially overtime during their hospital stay. By longitudinal tracking of such data, we aim to— (1) determine time trends in hematological parameters indicative of pathophysiological features of severe dengue, viz, plasma leakage (rising hematocrit) and thrombocytopenia, (2) determine the association of the longitudinal patterns in atypical lymphocyte and large immature cell with time trends in platelet counts and hematocrit.

2. Material and Methods

Retrospective data analysis was done on (n = 79) consecutive dengue patients hospitalized whom complete medical records were available. Enzyme - linked immunosorbent assay confirmed the diagnosis in all the

cases for both NS1 antigen and immunoglobulin M antibody. We anonymized the data before the statistical analysis. The Institutional Ethics Committee approved the study (EC/NEW/INST/1527/2022/11/37). The data collected included clinical features from case sheets and hematological parameters from medical records from admission to discharge. Dengue severity is stratified into three categories according to the World Health Organization (WHO) 2009 classification, viz, dengue fever without warning signs (DFWOWS), dengue fever with warning signs (DFWWS), and severe dengue. The hematological parameters were measured using Mindray BC - 5300 5 - part coulter. Hematological parameters that were longitudinally tracked during the hospital stay included platelet parameters (platelet count, mean platelet volume (MPV), platelet distribution width (PDW), leukocyte parameters (atypical lymphocyte, LICs, differential leukocyte count—neutrophil, lymphocyte, total leukocyte count, and red blood cells (RBC) parameters (RBC count, hematocrit, and hemoglobin concentration).

The data consisted of repeated measures of hematological parameters. Linear mixed models were used for the regression analysis. A linear mixed model was fit for every parameter to assess the time trend and its interaction with the disease severity. Regression coefficients from the model output were used to estimate the parameters' daily rise/fall trends. A parsimonious mixed model was also fit to predict platelet count from the hematological parameters. Statistical analysis was done using RStudio software, and mixed models were fit using the LME4 package.

A false discovery rate was used to adjust the p - values for multiple comparisons, and the adjusted p - value of 0.05 was used for statistical significance. We also report 95% confidence intervals (CI) for the regression slopes to indicate the precision of the model results.

3. Results

The clinical and demographic features of the participant cohort are summarized in Table 1. Of the total sample size of 79, only three were in the severe dengue category. Hence, the severe group was excluded from the inferential statistics because of the small sample. All the inferential statistics reported in the present work reflect the trends in the nonsevere dengue only, that is, DFWOWS and DFWWS. Table 2 shows the summary statistics of the hematological parameters. For continuous variables, mean ± standard deviation (SD) is shown, while for categorical data, frequencies are presented.

No. of patients	*DFWOWS*[†]	*DFWWS*[‡]	*Severe dengue*
N = 79 (females = 29)	*N = 40 (females = 14)*	*N = 36 (females =13)*	*N = 3 (females = 2)*
Age	19.9 ± 14.6	16.1 ± 14.1	5.3 ± 4.0
Age composition			
Age <12 years	13	17	3
Age ≥12 years	27	19	–
Clinical features			
Fever	39	35	3
Nausea/vomiting	19	24	3
Rash	3	7	0
Headache	10	3	1
Body pains	10	7	0
Abdominal pain	1	22	2
Edematous gall bladder wall	0	12	2
Abdominal tenderness	0	3	0
Mucosal bleeding	0	3	1
Ascites	0	14	2
Pleural effusion	0	13	2
Hepatomegaly/ splenomegaly	0	14	3
AST/ ALT >1000	0	0	3

[†]DFWOWS, dengue fever without warning signs; [‡]DFWWS, dengue fever with warning signs

	DFWOWS†	DFWWS‡	Severe dengue
Platelet count (×10³ cells/mm³)	121.1 ± 102.4 (minutes = 15)	111.0 ± 105.7 (minutes = 10)	102.3 ± 77.2 (minutes = 15)
MPV (fL)	9.4 ± 1.2 (minutes = 6.2)	9.2 ± 1.1 (minutes = 6.1)	9.5 ± 1.2 (minutes = 6.6)
PDW	16.6 ± 0.7 (minutes = 14.9)	16.7 ± 0.8 (minutes = 15.1)	16.9 ± 0.7 (minutes = 15.8)
Total WBC count (×10³ cells/mm³)	6.1 ± 3.8 (minutes = 1.55)	5.4 ± 3.3 (minutes = 0.95)	7.2 ± 3.3 (minutes = 2.59)
Differential count			
Neutrophil	47.1 ± 17.4 (minutes = 11)	43.3 ± 17.9 (minutes = 4)	33.3 ± 12.3 (minutes = 15)
Lymphocyte	43.1 ± 17.1 (minutes =4)	46.7 ± 18.1 (minutes = 8)	55.2 ± 10.7 (minutes = 30)
NLR	1.7 ± 2.3 (minutes = 0.13)	1.4 ±1 .6 (minutes = 0.09)	0.7 ± 0.5 (minutes = 0.2)
Monocyte	7.7 ± 3.1 (minutes = 0)	7.4 ± 3.1 (minutes = 1)	10.4 ± 4.0 (minutes = 5)
Atypical lymphocytes	1.7 ± 1.5 (minutes = 0)	1.9 ± 1.7 (minutes = 0.1)	2.6 ± 1.4 (minutes = 0.6)
LIC	0.9 ± 1.3 (minutes = 0)	1.6 ± 2.3 (minutes = 0)	1.0 ± 2.3 (minutes = 0)
Hematocrit	40.3 ± 6.4 (minutes = 26.9)	38.3 ± 5.9 (minutes = 23.4)	35.7 ± 4.6 (minutes = 29.7)
Hemoglobin (gm/dL)	13.3 ± 2.0 (minutes = 8.1)	12.7 ± 2.1 (minutes = 6.5)	11.9 ± 1.6 (minutes = 9.8)
RBC count (×10⁶ cells/mm³)	5.0 ± 0.7 (minutes = 2.94)	4.7 ± 0.8 (minutes = 2.47)	4.3 ± 0.6 (minutes = 3.53)

†DFWOWS, dengue fever without warning signs; ‡DFWWS, dengue fever with warning signs. For each parameter, mean and standard deviation are shown (mean ± SD). The lowest/nadir value (minute) for the same is also shown in the parenthesis

Platelet Parameters

The time trends of platelet parameters are shown in Figure 1. The platelet counts increased in the aggregated participant cohort (p < 0.001***) at a rate of 11524 cells/ mm3/day [(95% CI) = (5573, 17115) cells/mm3] approximately. The platelet count increase over time is significantly less in DFWWS when compared to DFWOWS (p = 0.001***). The rate of increase in platelet count is lesser in the DFWWS group by 21262 cells/mm3/day [95% CI = (−29502, −13080)]. In contrast, the average platelet count is not significantly different between the groups [p = 0.157|95% CI = (−10796, 68659)].

Mean platelet volume (MPV) has no significant trend over time [(p = 0.609|95% CI = (−0.04, 0.06)]. MPV is not different between the groups [(p = 0.181|95% CI = (−0.79, 0.14)].

In contrast, there is a significant interaction between the duration of hospital stay and the severity of dengue in affecting the value of MPV, with a difference of 0.08 femtolitre (fL) between the groups [(p = 0.04**, 95% CI = (0.012, 0.16)].

Platelet distribution width (PDW) is a stable parameter with no significant trend over time [(p = 0.553|95% CI = (−0.016, 0.061)], groups [(p = 0.571|95% CI = (−0.21, 0.41)] or interaction effect [(p = 0.571|95% CI = (−0.033, 0.074)].

Lymphocyte significantly increased over time [p < 0.001***|95% CI = (2.076, 3.626)]. The increase was greater in DFWWS when compared to DFWOWS [p = 0.007***|95% CI = (0.525, 2.737)]. Mirroring the lymphocyte, neutrophil declined over time [p = 0.002***|95% CI = (−4.213, −2.582)], with the decline greater in DFWWS when compared to DFWOWS [p = 0.033**|95% CI = (−2.315, −0.097)]. Total WBC count also increased with time [p = 0.008***|95% CI = (0.083, 0.411)], while the trend was nonsignificant over the group (p = 0.839|95% CI = (−1.621, 1.446)] and interaction effects [p = 0.183|95% CI = (−0.414, 0.06)]. The neutrophil - lymphocyte ratio (NLR) decreased over time [p = 0.002***|95% CI = (−0.561, −0.334)], with nonsignificant difference between the groups [p = 0.379|95% CI = (−1.063, 0.39)] and interaction effect (p = 0.105|95% CI = (−0.018, 0.293)].

RBC Parameters

The time trends in RBC parameters are shown in Figure 4. With multivariate analysis, hematocrit was found to have no significant trend over time [p = 0.172|95% CI = (−0.42, 0.03)], groups [p = 0.189|95% CI = (−4.132, 0.643)] or interaction effect [p = 0.189|95% CI = (−0.5, 0.116)]. In contrast, univariate analysis revealed a significant fall in hematocrit over time [p = 0.001***|95% CI = (−0.43, −0.14)]. Subgroup analysis revealed a greater fall of hematocrit in DFWWS [p = 0.044**, −0.185, 95% CI = (−0.35, −0.003)] when compared to DFWOWS [p = 0.006***, −0.398|95% CI = (−0.643, −0.181)].

On multivariate analysis, RBC count fell significantly over time [p = 0.015**|95% CI = (−0.058, −0.006)], while there was no significant difference between the groups [p = 0.644 | 95% CI = (−0.35, 0.21)] or interaction effect [p = 0.243|95% CI = (−0.057, 0.015)]. The univariate analysis also revealed a fall in RBC count over time [p = 0.001***|95% CI = (−0.062, −0.023)]. Subgroup analysis revealed a more remarkable fall in RBC count in DFWWS [p= 0.006***, −0.032|95% CI = (−0.055, −0.008)] when compared to DFWOWS [p = 0.001***, −0.053|95% CI = (−0.081, −0.027)].

Hemoglobin decreased significantly with time [p = 0.001*** | 95% CI = (−0.162, −0.025)], while there was no significant trend for severity [p = 0.241|95% CI = (−1.374, 0.313)] or interaction effect [p = 0.436|95% CI = (−0.14, 0.059)]. Similar results were found on univariate analysis with a fall in hemoglobin over time [p = 0.001***|95% CI = (−0.165, −0.064)]. Subgroup analysis revealed a fall in hemoglobin in both the groups DFWOWS [p = 0.009***, −0.093|95% CI = (−0.154, −0.032)] and DFWWS [p = 0.001***, −0.137|95% CI = (−0.215, −0.059)].

Association between Platelet Counts and Atypical Lymphocytes and Other Hematological Parameters

The fall and rise in the platelet count was closely mirrored by the rise and fall of ATY with higher values of ATY associated with lower values of platelets as shown in Figure 5 [p = 0.001***, regression coefficient = −14.64, 95% CI = (−20.747, −8.742)]. The regression coefficient value indicates that every 1% increase in atypical lymphocyte percentage is associated with a decrease in platelet count by

16, 963 cells/mm3. This suggests that the ATY may play a causal role in the platelet count fluctuations in dengue. A similar albeit weaker relation was found between platelet count and LIC, with a platelet count fall by 6, 680 cells/mm3 for every 1% rise in LICs as indicated by the regression coefficient [p = 0.006***, regression coefficient = −6.679|95% CI = (−11.4, −2.096)].

Multivariate regression analysis using linear mixed - effects models indicates that the following parameters are significantly associated with platelet count in dengue patients in the best parsimonious model— ATY (p = 0.007***), hematocrit (p = 0.004***), neutrophil% (p = 0.002***), lymphocyte (p = 0.002***), and total WBC count (p < 0.001***), the interaction effect between neutrophil and severity (p = 0.017**) and lymphocyte and severity (p = 0.002***).

4. Discussion

The present study was conducted to longitudinally track the hematological parameters in hospitalized dengue patients and explore the predictors of platelet counts, focusing on the ATY and large immature cell fraction for which the data from 79 patients were analyzed. The clinical profile and complications of dengue are thought to be induced by several mechanisms, 1, 4 involving cross - reacting adaptive immunity best illustrated by the “antibody - dependent enhancement” pathway. It is caused by nonneutralizing cross - reactive antibodies produced during a prior dengue infection by a different virus serotype. These antibodies are thought to promote the entry of the virus into FC - receptor - bearing cells wherein the virus propagates unimpeded, away from the fury of host immunity, with high viral load and the consequent inflammation provoked by disorganized cytokine response, vascular damage occurs, resulting in many pathological features. The vascular damage is known to compromise capillary integrity and cause plasma leakage from the capillaries into the tissues. The plasma leakage results in the rise of hematocrit. Antibody - mediated peripheral destruction and diminished production of platelets cause thrombocytopenia.

The hematocrit peaks in the critical phase and falls as the patient progresses into recovery, and leaked fluids are resorbed back into the circulation.

Similarly, the platelets attain the nadir in the critical phase and rise afterward.1, 5 We found that platelet count increased during the hospital stay, with the increase slower in DFWWS compared to DFWOWS. At the same time, the hematocrit (and other RBC parameters) decreased concurrently, suggesting that most of the patients are past the peak severity and are progressing from the critical stage to the recovery stage of dengue. Greater changes in the parameters in the DFWWS group are consistent with more severe disease in this group compared to the DFWOWS group.

Atypical lymphocytes fluctuated in lock - step with the platelet count but in an anticorrelated manner with lower platelet counts whenever the atypical lymphocytes increased and vice versa. We interpret this finding to suggest that atypical lymphocytes play a potentially causal role in the decline of platelet counts in dengue. The anticorrelated nature of the longitudinal trend strengthens the case for our conjecture regarding the causal association between them. Jampangern et al.200713 made an implicit suggestion regarding the identity of the atypical lymphocytes as they reported a significant positive correlation between atypical lymphocytes and a cluster of differentiation 19 (CD19) + B cells. In contrast, other studies14 have reported the presence of T cell markers (CD2 and CD7) on atypical lymphocytes. We conjecture that atypical lymphocytosis reflects the heightened and misdirected adaptive immune response to dengue characterized by the triad of crossreacting B and T cells, cytokine storm, and autoimmunity leading to tissue damage causing dengue pathophysiology.

Large immature cells (LICs) have a qualitatively similar but quantitatively weaker relationship with platelet count. A rise in LIC indicates increased release from the bone marrow of immature cells of both lymphoid/ myeloid lineage and can occur in infections and systemic inflammatory response conditions. We conjecture that fluctuations of humoral response (atypical lymphocytes) on the way to recovery induce corresponding changes in platelet counts and LIC, accounting for the anticorrelated nature of the relationship between platelets/hematocrit and atypical lymphocytes/LIC we show. Total WBC count increased over time consistent with recovery from critical phase induced fall in total WBC count. Virus - induced inhibition of myeloid progenitor cells has been proposed to explain the leucopenia in dengue. We interpret the rising WBC count in our data to indicate the recovery from the leucopenic dengue response. The neutrophil decreased while the lymphocyte increased, explaining the fall in the NLR in the cohort. The pattern of changes in neutrophils and lymphocytes (therefore, the NLR) we report is consistent with what is usually seen in viral infections.

5. Conclusion

The data from the present study offers a snapshot of changes in hematological parameters in the recovery phase following the peak of the rise/fall of cell counts. Nevertheless, by tracking their longitudinal trends, the data support the association between atypical lymphocytes and the pathognomonic hematological changes of dengue, viz, platelet count and hematocrit. Our study also supports the predictive potential of ATY and LIC in dengue. However, there is a paucity of effective prognostic markers for the cardinal pathophysiological feature of dengue, viz, vascular leakage and its severity. Future studies could address this deficit in our current understanding. Early prediction of the cardinal pathology can open new avenues for intervention to mitigate and reverse the incipient pathology of the disease in its tracks.

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Authorship Statement

RamadeviPeraka—conceptualization of the study, collection of data, critical review, and Editing of the manuscript; Aditya Koppula—analysis of the data, interpretation of results, and manuscript writing; Shalini MB—collection of data, critical review of the manuscript; Mani mekhalaParsa— conceptualization of the study and critical review of the manuscript.

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